

CHAPTER II

MATERIALS AND METHODS

2.1 Sample collection

Cases diagnosed as PBL and DLBCL were retrieved from the archives of the Departments of Oral Pathology of the Universities of Pretoria and Limpopo, South Africa. Only cases affecting the oral cavity were included in this series. This was a retrospective study on archival material and patient records. Ethics approval was received from the Research Ethics committee of the Faculty of Health Sciences, University of Pretoria: number 104/2008.

2.2 Clinical features

The clinical features of all cases retrieved for this study were noted from the histology request forms. When available the HIV status of the patients was recorded. Many patients presented for the first time with an intra-oral tumour or ulcer without any previous medical work-up. Very little medical history accompanied the histology request forms from where we retrieved clinical information for this study. After diagnosis of the lesions, most patients were lost to us for further study.

2.3 Microscopic features

The microscopic features of all confirmed cases of PBL were evaluated on thin (2µm) haematoxylin and eosin (H&E) stained sections and described in detail. Each case was classified according to its morphology as either 'PBL of the oral mucosa type' or as 'PBL with plasmacytic differentiation' using the criteria as described previously^{56, 92, 93} and also referred to in the 2008 WHO classification⁹. 'PBL of the oral mucosa type' was defined as a monotonous proliferation of large lymphoid cells with immunoblastic features with little or no plasmacytic differentiation and 'PBL with plasmacytic differentiation' was defined as immunoblasts and plasmablasts with more differentiation towards

mature plasma cells. HIV and EBV status was not taken into consideration for the morphologic classification in our study.

2.4 Immunohistochemistry

CD45, CD20, CD79 alpha, CD138, CD38, CD3, Ki-67 (MIB-1), MUM1 PROTEIN, IMMUNOGLOBULIN LIGHT CHAINS (KAPPA AND LAMBDA) and ALK protein

Immunohistochemical investigations were performed on three micrometer sections cut from formalin fixed paraffin embedded (FFPE) tissue blocks. Sections were de-paraffinized in two changes of fresh xylene for ten minutes each, re-hydrated with two changes of absolute ethanol, followed by 90%, 70% and 50% graded alcohol solutions respectively and washed with distilled water. Endogenous peroxidase activity was blocked by incubating the sections to undergo heat induced epitope retrieval (HIER) in a high pH buffer of a 3% aqueous solution of hydrogen peroxide for six minutes at 37°C. Blocking of endogenous peroxidase activity for the sections to undergo low pH HIER were performed after HIER. HIER in citric acid buffer, pH 6, was performed on the sections for CD45, CD20, CD79 α , CD138, CD38 and ALK protein and HIER in ethylene diamine tetra-acetic acid disodium salt (EDTA) buffer, pH 8.00 was performed for antibodies CD3, Ki-67, MUM1 protein and immunoglobulin light chains (kappa and lambda). HIER was performed using the Pascal, a pressurized heating chamber from Dako (DakoCytomation Denmark A/S, Produktionsvej 42, DK-2600, Glostrup, Denmark). The sections were treated at 120°C for 16 seconds according to the guidelines of the manufacturer. When the pressure was 0 psi in the Pascal it was opened and the sections were removed and cooled at room temperature for 20 minutes. The sections were rinsed in six changes of fresh distilled water and incubated in phosphate buffered saline buffer (PBS) (Sigma P4417, Sigma-Aldrich Chemie GmbH, P O 1120, 89552, Steinheim, Germany 49-7329-970) for ten minutes at room temperature.

The sections on which low pH (citric acid) HIER was performed were incubated with the following monoclonal mouse anti human ready to use

(RTU) antisera from Dako (Dako Cytomation Inc. 6392 Via Real, Carpintera, California 93013 USA) i.e. CD45 [Leucocyte Common Antigen Clone 2B11 and PD7/26 (Dako N1514)], CD20cy [clone L26 (Dako N1502)], CD79 α [clone JCB117 (Dako N1628)] and a 1:50 dilution of CD138 anti-serum [Clone MI15, (Dako M7228)] for 20 minutes at 37°C. The sections for ALK protein [CD246 Clone ALK1 (Dako IR641)] were incubated for 60 minutes at room temperature. A section of each case was also incubated in a 1:100 dilution of monoclonal mouse anti human CD38 [clone SPC32, (NCL-L-CD38-290) Novocastra Laboratories Ltd., Balliol Park West, Benton Lane, Newcastle Upon Tyne NE12 8EW United Kingdom] for 35 minutes at 37°C.

A section of each case, on which high pH HIER was performed, was incubated with RTU monoclonal mouse anti human CD3 [Clone PS1 (Novocastra RTU-CD3-PS1)] and Ki-67 [Clone MM1 (Novocastra RTU-Ki-67-MM1)], for 40 minutes at 37°C and in a 1:25 dilution of monoclonal mouse anti human MUM1 protein [clone MUM1p (Dako M7259)] for 60 minutes at room temperature. The sections for immunohistochemical investigation of kappa and lambda were first incubated in Protein Block Serum (Dako X0909) for ten minutes at room temperature to quench background staining before incubation with 1:100 monoclonal mouse Kappa light chain [Clone kp-53, (Novocastra NCL-KAP), Leica Microsystems] and 1:200 monoclonal mouse Lambda light chain [Clone HP-6054, (Novocastra NCL-LAM)] antibodies for 60 minutes at room temperature respectively.

All of the abovementioned sections were then incubated in Dako Envision+™ System Labelled Polymer, anti mouse, HRP [(horse radish peroxidase) Dako K4001], for 30 minutes at room temperature and rinsed in three changes of fresh PBS for five minutes each. The antigen complex was visualised by incubating the sections in AEC+ substrate chromogen (Dako K3469) for four minutes at 37°C, washed in distilled water and counterstained in Mayer's Haematoxylin for two minutes at room temperature. The sections were blued in tap water, rinsed in distilled water and mounted with Dako Faramount Aqueous Mounting Media (Dako S3025).

Strong red-brown granular staining, either on the cell membrane (CD45, CD3, CD20, CD38, CD138), cell nucleus (Ki-67) or in the cytoplasm of the tumour cells (MUM, ALK, CD79a, kappa and lambda) was regarded as positive. Photomicrographs (400X magnification) were taken of representative areas with a Leica DMD108 digital microimaging microscope. The image is displayed to a monitor. At least 500 tumour cells were then manually counted and evaluated in each case. Positivity was recorded, based on the percentage of positive staining tumour cells as follows:

- Focal: when less than 20% of cells in a given case stained positive,
- Intermediate: when 20-70% of cells stained positive
- Diffuse: when more than 70% of tumour cells stained positive.

Only tumour cells were evaluated in each case. Because the nature of the plasmacytic cells, neoplastic or reactive, was unknown, these cells were discarded from the evaluation of the immunohistochemical analysis.

Light chain restriction was defined as tumour cells staining for one light chain marker only (kappa or lambda) with no positive staining in any tumour cells for the other light chain.

2.5 *In Situ* Hybridisation for HHV-8 and EBV

2.5.1 HHV-8

Three micrometer sections of FFPE tissue blocks were cut, mounted onto Histobond^R slides (Paul Marienfeld GmbH & Co. KG Am Wöllerspfad 4 97922 Lauda-Königshofen, Germany) and incubated at 55 °C overnight. The sections were dewaxed in two changes of fresh Xylene, hydrated in two changes of absolute alcohol, 70% alcohol and three rinses of distilled water for three minutes each. HIER in citric acid buffer, pH 6, was performed using the Pascal, a pressurized heating chamber from Dako (DakoCytomation Denmark A/S, Produktionsvej 42, DK-2600, Glostrup, Denmark). The sections were treated at 120°C for 14 seconds according to the guidelines of the manufacturer. When the pressure was 0 psi in the Pascal it was opened and the sections were removed and cooled at room temperature for twenty minutes. The sections were rinsed in three changes of fresh distilled water for three minutes each, incubated in 0.01% pepsin (Sigma P6887, Sigma-Aldrich-Chemie. GmbH, P.O. 1120, 89552 Steinheim, Germany) in 0.2M hydrochloric acid for 11 minutes at room temperature and rinsed in three changes of distilled water for five minutes each. The slides were dehydrated in 95% and 100% alcohol for three minutes each and air dried.

Twenty microliter of Human Herpes virus (type 8) fluorescein labelled oligonucleotide cocktail probe solution designed to hybridise with a small transcript, designated T1.1 mRNA (Novocastra, NCL-HHV-8, Leica Biosystems Newcastle Ltd, Balliol Business Park West, Benton Lane, Newcastle Upon Tyne NE12 8EW, UK) was added to each slide, cover slipped and sealed with Fixogum rubber cement (PoseidonTM RFTM Marabuwerke GmbH & Co. KG, Asperger Strabe 4, D 71732 Tamm, Germany). Denaturing was performed at 65°C for 15 minutes to block endogenous alkaline phosphatase activity followed by hybridisation at 37°C for two hours using a StatSpin ThermobriteTM (Abbott Molecular, 3100 Woodcreek Dr. Downers Grove, 1L 60515 USA).

The cover slips were drained off in Tris Buffered Saline (TBS) [50mM Tris/HCl, 150mM NaCl pH 7.6) containing 0.1% Titron-X-100 (Bio Rad Laboratory, 2000 Alfred Nobel Drive, Hercules CA 94547)] for three minutes.

A post hybridisation stringency wash in 1:50 dilution of ready to use Dako stringent wash concentrate (Dako S3500) was performed at 55 degree Celsius for twenty minutes. The slides were rinsed in TBS containing 0.1% Titron-X-100 for three minutes.

To reduce background staining slides were placed on an incubation tray and the sections were covered with 100 µl of normal rabbit serum (Novocastra NCL-R-Serum) diluted 1:5 in TBS containing 3% w/v Bovine Serum Albumin (BSA) (Sigma A7906) and 0.1% Titron-X-100 and incubated at room temperature for ten minutes.

Detection was performed using Novocastra's *in situ* hybridisation detection kit (NCL-ISH-D). The blocking solution was tipped off and the slides incubated in 1:200 alkaline phosphatase-conjugated antibody to fluorescein isothiocyanate (FITC) (affinity-isolated rabbit F(ab') in 100mM Tris Buffer, 50mM MgCl₂, 0.1 mM ZnCl₂, 4% w/v BSA, stabiliser and preservative, pH 7.5) in TBS containing 3% w/v BSA and 0.1% v/v Titron X-100 for 30 minutes at room temperature. The slides were then washed in two rinses of TBS for three minutes each and thereafter in alkaline phosphatase substrate buffer (AFSB) (100mM Tris/HCl, 50mM MgCl₂, 100mM NaCl pH 9.0) for five minutes.

The sections were covered with 200 µl Enzyme substrate (5-bromo-4-chloro-3-indolylphosphate (BCIP) and Nitroblue tetrazolium (NBT) in dimethylformamide solution) diluted 1:100 in AFSB containing 1 µl Levamisole inhibitor per millilitre diluted enzyme substrate and incubated in the dark overnight.

The slides were washed in running water for five minutes, counterstained with Mayer's Haematoxylin for ten seconds and mounted with Dako Faramount aqueous mounting.

Black granular nuclear staining was accepted as positive for HHV-8. A Kaposi sarcoma section positive for HHV-8 was used as positive control and a brain section was used as negative control. Positive and negative controls were run with every batch of ISH for HHV-8.

2.5.2 EBV

Three micrometer sections of FFPE tissue blocks were cut, mounted onto Histobond^R slides (Paul Marienfeld) and incubated at 55° C overnight. The sections were dewaxed in two changes of fresh xylene, hydrated in two changes of absolute alcohol, 70% alcohol and three rinses of distilled water for three minutes each. The sections were placed on an incubation tray and covered with ready to use Proteinase K (Dako S3020) for three minutes at room temperature. The slides were then washed in two changes of distilled water for five minutes each, followed by 95% and 100% alcohol for three minutes each and air dried.

Twenty microliter of EBV fluorescein labelled oligonucleotide cocktail probe solution for the detection of mRNA (Novocastra, NCL-EBV, Leica Biosystems) sequences in EBV-encoded RNA (EBER) transcripts was added to each slide, cover slipped and sealed with Fixogum rubber cement (PoseidonTM). Denaturing was performed at 65°C for fifteen minutes to block endogenous alkaline phosphatase activity followed by hybridisation at 37°C for two hours using a StatSpin ThermobriteTM (Abbott Molecular). The cover slips were drained off in TBS containing 0.1% Titron-X-100 for three minutes and washed in three changes of fresh TBS containing 0.1% Titron-X-100 for three minutes each.

To reduce background staining slides were placed on an incubation tray and the sections were covered with 100µl of normal rabbit serum (Novocastra NCL-R-Serum, Leica Biosystems) diluted 1:5 in TBS containing 3% w/v BSA (Sigma A7906) and 0.1% Titron-X-100 and incubated at room temperature for ten minutes.

Detection was performed using Novocastra's ISH detection kit (NCL-ISH-D Novocastra, Leica Biosystems) according to the manufacturer's guidelines.

The blocking solution was tipped off and the slides incubated in 1:400 alkaline phosphatase-conjugated antibody to FITC (affinity-isolated rabbit F(ab') in 100mM Tris Buffer, 50mM MgCl₂, 0.1 mM ZnCl₂, 4% w/v BSA, stabiliser and preservative, pH 7.5) diluted 1:400 TBS containing 3% w/v BSA and 0.1% v/v Titron X-100 for thirty minutes at room temperature. The slides were then washed in two rinses of TBS for three minutes each and thereafter in AFSB buffer for five minutes.

The sections were covered with 200µl Enzyme substrate (NBT/BCIP) diluted 1:200 in AFSB containing 1µ Levamisole inhibitor per millilitre diluted enzyme substrate and incubated in the dark overnight.

The slides were washed in running water for five minutes, counterstained with Mayer's Haematoxylin for ten seconds and mounted with Dako Faramount aqueous mounting media.

Black granular nuclear staining was accepted as positive for EBV. A nasopharyngeal carcinoma section positive for EBV was used as positive control and a brain section was used as negative control. Positive and negative controls were stained with every batch of *ISH* done for EBV.

2.6 Fluorescence *in situ* hybridisation

2.6.1 FISH technique

Four micron sections of FFPE tissue blocks were cut, mounted on Super Frost plus slides (Menzel-Gläser, Gerhard Menzel GmbH, Saarbrückener Str.248 D-38116 Braunschweig, Germany), dried overnight at 56°C and matured at room temperature for two days. The sections were deparaffinized in two changes of fresh xylene for 15 minutes each followed by two rinses in absolute alcohol for five minutes each at room temperature and air dried. The slides were incubated in 0.2 N hydrochloric acid (HCl) for 20 minutes at room temperature, rinsed in pure water for three minutes followed by three minutes in two times saline sodium citrate (SSC) buffer and air dried. Pre-treatment of the slides were done in a 0.1M sodium thiocyanate (NaSCN) for 30 minutes at 80°C followed by a one minute rinse in pure water and two washes in two times SSC buffer for five minutes each at room temperature. Digestion of the sections was done in a pre-warmed 0.05% Pepsin (Pepsin, Roche Diagnostics GmbH Mannheim, Germany) in 0.1N HCl at 37°C for 60-90 minutes. This was followed by two washes in two times SSC for five minutes each at room temperature. Slides were fixed in a 1% formaldehyde solution for ten minutes at room temperature, rinsed twice in two times SSC buffer and dried on a slide warmer.

FISH analyses using the following probes from Vysis (Vysis Inc, Downers Grove, Illinois, USA) were done on separate sections of each case: LSI *IGH* dual colour BA rearrangement probe, LSI *MYC* dual colour BA rearrangement probe, LSI *BCL6* dual colour BA probe, LSI *IGH/MYC* CEP 8 tri-colour, dual fusion translocation probe, LSI *CCND1/IGH* dual colour dual fusion translocation probe and LSI *IGH/BCL2* dual colour dual fusion translocation probe. The probes were prepared according to the manufacturer's instructions. The sections were cover slipped with probe solution and sealed with Marabu Fixogum Rubber cement (Marabuwerke GmbH & Co. Tamm Germany). The sections were protected from light from hereon. Denaturation and hybridisation was performed in a thermocycler (Thermobrite, Abbot

Molecular Illinois, USA). Slides were denatured at 75°C for five minutes and then hybridised overnight at 37°C.

The rubber cement was removed and the cover slips washed off in 2 x SSC. A post hybridisation wash was performed on the slides at 76°C for four minutes in 0.05% Tween/2 x SSC and counterstained in 200 ng/ml 4', 6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Merck, Darmstadt, Germany) for 15 minutes at room temperature. The sections were rinsed in 0.05% Tween/2 x SSC and cover slipped with Vectashield mounting media for fluorescence (Vector Laboratories, Inc Burlingame, CA 94010), sealed with fixogum rubber cement and evaluated with a Nikon Eclipse E1000 microscope equipped with a spectrum- green, orange, aqua, DAPI and triple band filter.

2.6.2 Criteria for positive FISH results:

IGH-BA rearrangement probe (14q32.3 LSI IGH)

In a normal cell hybridised with the *IGH*-BA probe, the expected signal pattern is two yellow fusion signals per nucleus. In a cell with an *IGH* translocation breakpoint, one spectrum green (LSI 5' flanking probe), one spectrum orange (LSI 3' flanking probe) and one yellow fusion signal pattern should be observed. Slides were scored based on knowledge of background levels of BA patterns in normal lymphoid tissue using at least 100 nuclei counts and verified by three individual analysts. The cut-off value for a positive result with the IGH-BA probe in this study was 5%.

IGH/MYC, CEP 8 tri-colour, dual fusion translocation probe [t(8;14)(q24;q32)]

In a normal cell hybridised with this probe, the expected pattern is two spectrum aqua (chromosome 8 CEP), two spectrum orange (*MYC*-gene), and two spectrum green (*IGH*-gene) signals. A cell harboring the reciprocal t(8;14) with a 8q24 breakpoint within the *MYC* locus is expected to produce a pattern of one orange, one green, two orange/green fusions, and two aqua signals. If the cell contains a breakpoint very far 5' of *MYC*, a fusion on the

der(8) may not be visible or may be very weak, as little or no red probe target would remain on the der(8). Slides were scored based on knowledge of background levels of fusion patterns in normal lymphoid tissue using at least 100 nuclei counts, verified by three individual analysts. The cut-off value for a positive result with the t(8;14) probe in this study was 15%.

***MYC BA* rearrangement probe (8q24 LSI MYC)**

In a normal cell hybridised with the *MYC*-BA probe, the expected signal pattern is two orange/green fusion signals. In a cell with a translocation breakpoint, one green, one orange and one fusion signal pattern should be observed. Slides were scored based on knowledge of background levels of BA patterns in normal lymphoid tissue using at least 100 nuclei counts, verified by three individual analysts. The cut-off value for a positive result with the *MYC*-BA probe in this study was 5%.

***IGH/CCND1* dual colour, dual fusion translocation probe [t(11;14)(q13;q32)]**

A cell harboring a t(11;14) with breakpoints in the *IGH* locus on 14q32 is expected to have a signal pattern of two yellow fusions, one on each of the translocation derivative chromosomes 11 and 14 as well as one spectrum orange (*CCND1* on chromosome 11) and one spectrum green (*IGH* on chromosome 14) signal from the normal chromosomes 11 and 14. Slides were scored based on knowledge of background levels of fusion patterns in normal lymphoid tissue using at least 100 nuclei counts, verified by three individual analysts. The cut-off value for a positive result with the t(11;14) probe in this study was 15%.

***IGH/BCL2* dual colour, dual fusion translocation probe [t(14;18)(q32;q21)]**

A normal nucleus hybridized with the LSI *IGH/BCL2* probe shows two spectrum orange (*BCL2*) and two spectrum green (*IGH*) signals. In a nucleus harbouring a t(14;18), the most common pattern is one orange signal, one

green signal (representing the normal chromosome 14 homolog) and two yellow fusion signals representing the two derivative chromosomes resulting from the reciprocal translocation. Slides were scored based on knowledge of background levels of fusion patterns in normal lymphoid tissue using at least 100 nuclei counts, verified by three individual analysts. The cut-off value for a positive result with the t(14;18) probe in this study was 15%.

***BCL6* BA rearrangement probe (3q27)**

In a normal cell hybridised with the *BCL6* BA probe, the expected signal pattern is two yellow fusion signals. In a cell with a translocation breakpoint in the *BCL6* locus, one spectrum green, one spectrum orange and one fusion signal pattern should be observed. Slides were scored based on knowledge of background levels of BA patterns in normal lymphoid tissue using at least 100 nuclei counts, verified by three individual analysts. The cut-off value for a positive result with the *BCL6* BA probe in this study was 5%.

Selected images were captured using the Genus™ CytoVision 3.0 software (Applied Imaging).

2.6.3 General aspects of FISH analysis

Scoring of FISH results in FFPE slides required compensating for several potential artifacts. My approach in this study was to evaluate the signals in 100-200 tumour cells, depending on the availability of tumour cells optimal for evaluation and then to study them in detail in order to work out the percentages of signal patterns encountered in every case. Although this was very time consuming, the method resulted in optimal results which was 100% comparable between all three analysts involved in the study.

The possibility of false positive results was considered in each case. This depended very much on the type of probe used. The two probes of a fusion assay may come to lie accidentally close to one another, producing a false positive yellow-like fusion signal. This also holds true for the BA probe where great care should be taken when interpreting the distance between the orange

and green signals. This varies between different probes. An example of this was the IGH/CCND1 dual colour, dual fusion translocation probe (t(11;14)). Due to the gap between the two probes in the *IGH* probe set, the normal *IGH* loci may sometimes appear as two slightly separated green signals. This gap may in some instances also cause a slight separation of the orange and green signals on the der(11) chromosome. One way to overcome these problems is to have well-tested cut off criteria to mark a case as either positive or negative by doing enough negative control cases. In our study the cut-off value for a positive result with BA probes were 5% and the cut-off value for a positive result with the fusion probes were 15%.