CHAPTER 4 BIOPSY TECHNIQUES AND TISSUE HANDLING

4.1 Prebiopsy

4.1.1 Biopsy planning: interaction between clinician and pathologist[1]

In an optimal situation, discussion takes place between the surgeon, the treating haematologist/oncologist, the anatomical pathologist and the laboratory haematologist before biopsy. Knowledge of the clinical history and differential diagnosis allows planning of the most appropriate biopsy site and technique, special studies needed, and time and place of the biopsy. Review of the hemogram and blood film is recommended, and the issue of patient consent can be addressed if any tissue is to be kept for research or submitted to a tissue bank.

In reality, however, this ideal situation is often unobtainable, underscoring the importance of providing full clinical information to the pathologist.

4.1.2 Clinical details required on pathology request form[1–4]

The WHO classification is a clinicopathological system in which a detailed understanding of the clinical presentation is fundamental to the diagnosis (see Chapter 3). Almost universally, however, the clinician who performs the biopsy and submits the pathology request is a surgeon or interventional radiologist rather than the clinician responsible for clinical investigation and management. It is therefore essential that the managing clinician be separately identified on the request form and that the following information be made available prior to diagnosis.

Requirements include:

i Patient demographics

ii Clinician performing biopsy

iii Clinician responsible for patient investigation and management

iv Date of procedure

v Duration lymphadenopathy or other mass

vi Localised or generalised disease

vii Evidence of organomegaly

viii Other signs and symptoms, for example, constitutional symptoms

ix Relevant haematological findings

x Underlying disease or immunosuppression

- a Viral: HIV, HTLV, EBV
- b Autoimmune disease
- c Congenital immune disorder
- d Known cofactors (e.g. Helicobacter infection)

xi Provisional diagnosis

xii Site of biopsy

xiii History of previous lymphoma:

a Dates

b Site

c Previous diagnosis

d Previous treatment (e.g. transplantation).

e Treatment status (e.g. complete remission, partial remission, relapse)

Key point

There is a minimum amount of information that should be included on request forms. It is recommended that specific histopathology request forms be developed that include the information in Section 4.1.2, and that they be used generically in oncology (see suggested format in Figure 4.1).

Figure 4.1 Clinical request form

Clinical Request Information……………………. ………………………………

Surname ……………..First name …………… UR No. ……….. Sex …..DOB……..
Address ……………………………………………………………………………………
Name of clinician performing the biopsy …………………………….
Name of clinician managing the patient …………………………….

Current illness:

Disease duration:	……………………………….
Presenting complaint:	
Disease extent:	Unknown / Solitary / Localised / Generalised
Known sites of disease:	*Nodal sites: (indicate on diagram) or:* Specify: …………………………………… *Extranodal sites:* …………………………..
Organomegaly:	Unknown / Hepatomegaly / Splenomegaly Other: ……………………………….
Constitutional symptoms	Unknown / Yes / No
Relevant haematology:	Unknown / Specify:…………………………….
Provisional clinical Dx:	Unknown / NHL / Hodgkin lymphoma / Reactive Other: ……………………………….

Relevant past Hx:	Unknown / Nil / Autoimmune Disease / Medication Other: ……………………………
Immunosuppression:	Unknown / Viral / Congenital / Transplantation / Methotrexate Other: ……………………………
Previous lymphoid disease:	Unknown / Nil / Yes
Diagnosis	Specify:………………………………….
Date	………………………………….
Site	………………………………….
Stage	…………………………………
Laboratory:	………………………………….
Laboratory Ref. No.:	………………………………….
Treatment(s):	
Modality:	Specify: ……………………………
Completion:	Ongoing / Completed (date………….)
Response:	CR / PR / NR

4.2 Biopsy modalities

4.2.1 Fine-needle aspiration biopsy

Fine-needle aspiration (FNA) biopsy is a useful technique for the initial triage of lymphoproliferative disease and to obtain material for flow and other ancillary studies by the least invasive technique. It has a role in the diagnosis of metastatic tumours in lymph nodes[5,6] and may aid in distinguishing between reactive lymphoid hyperplasia and lymphoma when used in conjunction with flow cytometry FCM.[1,6–8]

Key point

Fine-needle aspiration (FNA) biopsy should not be used in the definitive diagnosis or subtyping of lymphomas, for which excision biopsy remains the definitive procedure.

Indications for FNA

i Triage of lymphadenopathy or a mass lesion, superficial or deep:

- a Haematolymphoid neoplasm versus other malignancy
- b Reactive lymphoid hyperplasia versus lymphoma

ii Staging

iii Monitoring for:

- a Residual disease
- b Recurrence
- c Tumour progression

iv As an adjunct to conventional biopsy

- a To obtains better cytological detail
- b To obtain fresh material for ancillary studies such as flow studies (FCM), cytogenetics, molecular studies, etc.

Guideline — Fine-needle aspiration (FNA) biopsy	Level of evidence	Refs
FNA is the biopsy investigation of choice in the initial triage of a possibly lymphomatous lesion, and should be accompanied by flow cytometry (FCM) studies.[1,6–8]	IV	9–17

Technique for FNA

To ensure high-quality preparations, a person experienced in FNA biopsy technique should perform this procedure.[18]

A 25 G or 23 G needle is manoeuvred to the capsule or edge of the target and then repeatedly and rapidly pushed into the area in question. Between six and 20 movements are usually made before the needle is removed and the contents expunged for triage. The procedure may be performed with or without aspiration.

FNA specimens must be triaged immediately. Direct smears are prepared and stained as for imprints (see Section 4.3.2) and a suspension is submitted for FCM. Other allocations are made according to the clinical circumstances (see Section 4.3.2). It is advisable for a cytopathologist or cytologist to attend the procedure to check adequacy of the biopsy, prepare the smears for optimal morphology and assist in triaging the specimen.

Key point

To optimise fine-needle aspiration (FNA) biopsy, it is preferable for a cytopathologist or cytologist to attend the procedure to check adequacy of the biopsy, prepare the smears, and assist in triaging the specimen.

Advantages and disadvantages

The **advantages** of FNA can be summarised as follows:

i Excellent triage tool

ii Good material for flow studies is readily obtained

iii Minimal invasiveness

iv Few complications:

- a Bleeding
- b Pneumothorax
- c Infection

v Rapid result

vi Inexpensive

vii Easy to perform

The **disadvantages** of FNA include the following:

It is inappropriate for **definitive** diagnosis and subtyping due to:

i Absence of architectural information seen in tissue sections

ii Absence of immuno-architectural information seen in immunostains of tissue sections[19]

iii Sampling problems

- a Partial lymphomatous infiltration
- b Composite lymphoma
- c Lymphoma with sparse neoplastic cells:

- Hodgkin lymphoma
- T-cell rich B-cell lymphoma

iv Technical limitations

a Air-drying artefact

b Blood contamination

c Smear artefact

d Necrosis

e Dry aspirates (fibrotic lesions)

v High level of diagnostic expertise is required

vi Test performance is significantly poorer than tissue biopsy

Test performance

Differentiation of lymphoma from hyperplasia

The unsatisfactory rate for FNA is reported to be 3–16%.[20]

Using cytomorphology alone, the accuracy of FNA in the diagnosis of malignant lymphomas is reportedly between 64% and 72%[9–12], with a false negative rate up to 12–14%.[10] Small lymphocytic proliferations in particular have a high false negative rate using FNA cytomorphology alone.

With the addition of FCM, the accuracy of lymphoma diagnosis has been claimed to be between 77% and 87%[11–14], and the false negative rate as low as 3.5–5%.[9–11] Many of the studies, however, did not define the 'gold standard' by which test accuracy was measured. In those series where combined FNA and FCM findings have been verified by subsequent histological biopsy, a test sensitivity of 80–83% has been claimed.[21,22] Accuracy may be significantly improved when dealing with recurrent disease.[13,23]

The test accuracy quoted above relates to studies that consist largely of B-cell tumours, since these are the most common forms of lymphoma in western society. They do not apply specifically to the diagnosis of Hodgkin lymphoma as in this disease, FCM findings are normal and the diagnosis depends entirely upon cytomorphology. The diagnostic accuracy of FNA in Hodgkin lymphoma by FNA ranges from 30% to 85–90%[15,24–26], while the accuracy of subtyping is poor[15,24], due partly to lack of architectural information in FNA samples. Similarly, the test accuracy may be lower for T-cell lymphomas as clonality cannot be directly assessed by FCM, and many T-cell lymphomas lack phenotypic aberrancy.

Lymphoma subtyping

In defining a specific lymphoma subtype or disease category, the accuracy of FNA cytomorphology alone has been reported to be between 37% and 64%[10,23], while the accuracy of FNA combined with FCM is reported to be between 77% and 84%.[7,10,12,13,23]

A more recent literature review of FNA combined with immunophenotyping[16] has documented a wide range of precise classification (from 18% to 100%), with histological confirmation of the cytological diagnosis varying from 10% to 100%.

Most series are small and many consist almost entirely of B-cell lymphomas, both primary and recurrent, often with a predominance of one disease subtype. A particular problem arises in follicular

lymphoma, where there are no established criteria for the subtyping or grading of follicular lymphomas in cytological preparations, and the method varies in the different series cited above.[10,15,27]

The test accuracy quoted above does not apply in Hodgkin lymphoma in which differentiation of the subtypes from each other and from various NHL simulants, including anaplastic large-cell lymphoma, T-cell lymphoma and T-cell rich B-cell lymphoma, may be exceedingly difficult even after examination of good histological tissue sections and an extensive panel of immunohistochemical stains. Surgical biopsy is recommended to confirm (or make) the diagnosis and to subclassify the disease.[15,17]

Guideline — Definitive tissue biopsy	Level of evidence	Refs
Tissue (as distinct from FNA) biopsy is essential for the primary diagnosis, subtyping and clinical management of lymphoma.	IV	7, 10, 13, 15, 27–29

Key point

It is acknowledged that in rare cases where the clinical circumstances preclude tissue biopsy, it may be appropriate to proceed to treatment with a lower standard of diagnostic proof.

4.2.2 Cytological specimens other than FNA

Protocols are needed in pathology services to ensure appropriate specimen delivery, handling and rapid triage of cytological specimens other than FNA, for example, effusions (see below).

4.2.3 Needle core biopsy

For many years, image-guided needle core biopsies (NCBs) have been applied to numerous organs with excellent results and few complications.[30–32] Following the widespread adoption of the WHO classification of lymphomas and ancillary studies, NCB is gaining increasing acceptance in the diagnosis and management of deeply situated lymphoma.[33–36]

Key point

In the presence of surgically accessible, superficial lymphadenopathy, needle core biopsy has little role in *primary* lymphoma diagnosis, since fine-needle aspiration is the optimal form of triage, and excision biopsy is the investigation of choice for definitive diagnosis.

In deeply situated lesions, however, CT or ultrasound-guided core biopsies have a number of advantages, and in many cases can provide definitive primary diagnosis of lymphoma. NCB nonetheless provides material inferior to surgical specimens, and a significant minority of cases will still require surgical biopsy for definitive diagnosis. Moreover, even when a specific lymphoma subtype can be diagnosed with confidence, the risk of sampling error ensures that this modality must always be inferior in quality to surgical biopsy.

Indications

i Staging

ii Monitoring

iii Residual disease[36]

iv Recurrence[33,35,36]

v Tumour progression[33,35,36]

vi Obtaining material for ancillary studies (e.g. flow cytometry, cytogenetics)[19]

vii In cases without surgically accessible peripheral disease

viii For the primary diagnosis and subtyping of lymphoma (surgical biopsy subsequently required in a minority of cases).[19,33–37]

NCB may be deemed unsuitable in the following circumstances due to the risk of significant morbidity:

i Clotting disorders, anticoagulant therapy

ii Pulmonary or hepatic hilar disease

iii Intraparenchymal lung disease

iv Para-aortic, para-caval nodes

v Aortopulmonary window disease

vi Lesions surrounded by bowel

Technique

A variety of biopsy guns are available, with both advancing and non-advancing needles. Needle sizes range from 12 G to 20 G.

Although a recent study of 211 cases failed to show a correlation of diagnostic accuracy with needle gauge[35], most cases in the study used 16 G and 18 G needles. Few 20 G samples were included in the sample. Although we could not find any literature to provide an evidence-based guideline, the consensus view of the diagnostic committee is that NCBs of 20 G or smaller diameter are prone to fragmentation and significant crush artefact.

Key point

In the absence of a higher level of evidence to the contrary, needle biopsies of 18 G or 16 G are preferable.

There are few data correlating morbidity with needle diameter.

Sufficient material should be obtained to allow for necessary ancillary studies.

In virtually all cases, FNA biopsy should be performed concurrently, as it adds little to the morbidity of NCB yet provides superior cytomorphology and is an excellent source of a cell suspension for FCM.

It is recommended that the CB specimen be triaged at the time of biopsy, either by the attending cytologist or pathologist, or by the surgeon or radiologist, and that the specimen for histology be placed in formalin as soon as possible to prevent drying.

Key point

Needle core biopsy performed for the diagnosis of suspected lymphoma should be accompanied by fine-needle aspiration and material for flow cytometry.

Advantages and disadvantages

Compared with FNA, NCB has the following **advantages**:

i Some (limited) architectural information is available[19]

ii Sampling error is reduced

iii Paraffin sectioning enables:

- a Paraffin section immunophenotyping[19,36]
- b Immuno-architectural assessment (using CD21, CD23 or CD35 for FDCs)
- c Paraffin tissue-based PCR and/or FISH[19]

NCB has the following **disadvantages**:

i Increased complications compared with FNA (up to 7.5% of cases)[34,35]

- a Haematoma[30]
- b Pneumothorax[30]
- c Local discomfort[36]
- d Vasovagal attacks[36]

ii Cytological assessment may be compromised by:

- a Crush artefact[35]
- b Loss of chromatin detail (may impart a ‘blastic’ appearance)[38]

Compared with surgical biopsy, NCB has the following **disadvantages**:

i Inadequate sample (up to 14%)[19,32,36]

- a Non-representative sample, for example, surrounding tissue
- b Diseases with few malignant cells, for example, T-cell rich B-cell lymphoma, Hodgkin lymphoma[19]
- c Diseases with zonal variability, for example, MALT lymphoma[19]
- d Insufficient material for ancillary studies

ii Morphological detail compromised

iii Crush artefact[35]

iv Necrosis or sclerosis may limit sampling[32]

v Nuclear smudge artefact may impart a 'blastic' appearance[38]

vi Architectural assessment limited

NCB has the following **advantages**:

i A general anaesthetic is avoided[35]

ii Hospitalisation time is short (mean one day)[35]

iii Low cost[35]

iv Well tolerated[36]

v Lower morbidity[19,35,36]

vi Less invasive[35]

Test performance

Differentiation of lymphoma from hyperplasia

Diagnostic accuracy of NCB is reportedly between 58% and 89% overall.[33,35,36] A recent series of 66 cases of cervicofacial lymphadenopathy, however, reported a sensitivity, specificity and accuracy of 98.5%, 100% and 98.7% respectively in differentiating lymphoma from lymphoid hyperplasia.[39]

Lymphoma subtyping

The same review of 66 patients reported successful primary diagnosis and subclassification in 80% of cases, obviating the need for surgical biopsy. Other studies have reported an accuracy of 75–85% with the aid of immunoperoxidase stains.[33,36] Diffuse B-cell lymphomas and follicular lymphomas predominate in many studies.

4.2.4 Endoscopic biopsy

Endoscopic biopsies provide morphological information similar to that of NCBs — including similar issues of crush artefact and sampling error. Immunophenotyping by immunohistochemistry is readily performed. There is limited quantitative information upon the efficacy of flow surface marker studies, but a number of studies have clearly demonstrated the value of flow studies in endoscopic biopsies.

4.2.5 Surgical biopsy of lymph node where lymphoma is suspected

This is discussed in Chapter 10.

4.2.6 Bone marrow aspirate and trephine

Indications

i Staging at initial diagnosis

ii Restaging following treatment

iii Assessment of minimal residual disease

iv Assessment of cytopenias in patients with an established diagnosis

v Rarely, for the primary diagnosis and subtyping of lymphoma in patients with no other accessible disease.

For **staging**, the result should be scored as positive (unequivocal cytological or architectural evidence of malignancy), negative (no aggregates or only a few well-circumscribed lymphoid aggregates), or indeterminate (increased number or size of aggregates without cytological or architectural atypia). The extent and the pattern of marrow involvement, along with the cell type, should be reported.

Assessment of **minimal residual disease** is carried out using one or more ancillary techniques. Flow cytometry (FCM) may demonstrate B-cell monoclonality or aberrant B-cell or T-cell phenotypes. In certain disease subtypes, immunostaining may detect low levels of tumour.

In cases with no morphological or immunophenotypic evidence of residual tumour, molecular studies for immunoglobulin heavy-chain gene, or T-cell receptor gamma rearrangement, may be performed. These are generally not carried out routinely, except in the setting of clinical trials where patients are being treated in subspecialised centres that have the appropriate expertise to perform the assays and interpret the results. In certain specific subtypes of lymphoma, specific oncogenes may be assayed, for example, polymerase chain reaction (PCR) for *bcl-1* in patients with mantle cell lymphoma, *bcl-2* in follicular lymphoma, or *c-myc* in Burkitt lymphoma.

In general, lymphoma patients are currently not being treated on the basis of detectable molecular disease post-cytotoxic therapy. This may change in the future, analogous to certain leukaemias such as acute promyelocytic leukaemia and chronic myeloid leukaemia.

The cause of cytopaenia**s** can be assessed. They may be due to marrow replacement with lymphoma, cytotoxic therapy, increased peripheral destruction, or development of secondary myelodysplastic syndrome/acute leukaemia in previously treated patients.

Bone marrow examination is not recommended for the **primary diagnosis** of lymphoma because of frequent histological discordance between marrow and other sites.[40–42]

Guideline — Requirements for bone marrow examination	Level of evidence	Refs
Bone marrow examination is not recommended for the primary diagnosis and specific subtyping of lymphoma, except in special circumstances.	IV	40–42

For certain types of lymphoproliferative disease that commonly present in the bone marrow, a definitive diagnosis may be made on this material alone. Examples include acute lymphoblastic lymphoma (ALL), small lymphocytic lymphoma (chronic lymphocytic leukaemia), prolymphocytic lymphoma, lymphoplasmacytic lymphoma and hairy cell leukaemia.

For most other types of lymphoma, definitive diagnosis will require excision biopsy of representative material from the primary disease site. Disease confined to the marrow is an obvious exception.

In some circumstances, bone marrow may be the only accessible site of disease. In such cases, a lower standard of diagnostic proof may be accepted by the treating clinician and the bone marrow used for primary diagnosis.

Technique

It is important that the procedure be carried out by haematologists (trained or in training), or other medical practitioners specifically trained in this technique.

Aspiration alone is not recommended. Ideally, triage occurs at the time and place of the procedure.

For bone marrow biopsies, direct aspirate smears (without anticoagulant) should be prepared at the time and place of biopsy. However, if that is not practical, the aspirate should be placed in an EDTA tube and films made within one to two hours. Two aspirate smears and one trephine imprint should be stained with one of the Romanowqsky stains. The stain recommended by the Royal College of Pathologists of Australasia is the ICSH stain (International Committee for Standards in Haematology). The smears should be stained within 24 hours. If that is not possible, they should be fixed in methanol and stained as soon as possible.

The ideal length of a core biopsy is 20 mm.[40] For staging, this should ideally be examined at 3–4 levels 0.10–0.2 mm apart. Such examination obviates the need for bilateral bone marrow biopsies.[41–43]

Ideally, triage occurs at the time and place of the procedure.

4.3 Transport, handling and triage of biopsy material

4.3.1 Transport of fresh excision biopsy tissues

Where lymphoma is suspected, all specimens should immediately be sent intact and unfixed in a closed sterile container to the laboratory (anatomical pathology) for triaging.[4] The specimen must be identified and accompanied by a detailed request form (see Section 4.1.2). Drying must be avoided. If paper or PVA pads are used, they should be moistened with physiological saline. If there will be any delay in transportation, the specimen should be floated in sterile physiological saline, Hanks solution, or RPMI 1640 culture medium. Immunofluorescence transport medium containing ammonium sulphate is not suitable.

Specimens may be transported at room temperature for up to two hours. For delays of 2–24 hours, they may be stored at 4°C or cooled on wet ice, but not allowed to freeze. Dry ice is not appropriate as it will freeze the specimen.

Specimens for conventional cytogenetics should be kept sterile and at room temperature, in RPMI 1640 (Section 4.3.4).

4.3.2 Laboratory handling and triage of fresh tissue

Tissue should be handled quickly to preserve morphology, antigens and cell viability. Specimens other than needle core biopsies should always be sliced to allow proper fixation. Drying must be avoided at all stages and each time tissue is sliced, it should be immersed in the appropriate fluid immediately. A written protocol should be available for specimen handling in each institution.[1,4,44–49]

Many centres have established tissue banks for prospective studies. It is recommended that this practice be encouraged.

Macroscopic description should include

i Patient identifiers (name, medical record number)

ii Organ or site

iii Received:

- a Fresh, in fixative or other fluid
- b Intact, sectioned, fragmented

iv Nature of biopsy (core, incisional, exisional, resection)

v Weight (for spleen and other organs)

vi Dimensions

vii Description of capsule and cut surface: (colour, consistency, necrosis, haemorrhage, nodularity)

Universal handling

As the WHO classification of lymphoma is based on a combination of morphology, immunophenotype, genetic features and clinical features, almost all specimens, including tissue, cytology specimens and bone marrow specimens, need to be divided to allow ancillary investigations. The constraints of specimen size, cost and service availability mean that the most appropriate ancillary tests need to be selected on a case-by-case basis, taking into account the information from the preoperative consultation and initial triage (intraoperative examination or FNA).

i. Slicing

Using a sterile technique, slice the lymph node or tissue cleanly with a sharp scalpel or razor blade, in 2 mm thick slices, perpendicular to the long axis so the poles are then available for ancillary studies that require fresh tissue.[4]

NCB specimens are rarely divided, except when required for triage.

Spleens should be sliced at 3–5 mm thickness, especially if removed for staging. Initial fixing of 10 mm thick slices in formalin may facilitate thin slicing.[4] Splenic lymph nodes should be dissected from the hilum and treated in the same way as lymph nodes.

Extranodal tissue and large resection specimens such as stomach, salivary gland, bowel, lung or other organs will require dissection and detailed description in addition to the above.[2] They should be submitted fresh and triaged for ancillary studies in the same manner as nodal disease.

ii. Make imprints

Surgical specimens: Make imprints or touch preparations of the freshly cut surfaces, taking care not to drag, squash or traumatise the tissue. Touch the prelabelled glass slide lightly to a freshly cut surface that is held face up to avoid blood draining down onto the slide. Some may be air-dried and Giemsa stained, formalin fixed and H&E stained, or alcohol-fixed and PAP stained. Imprints may be used for intraoperative diagnosis, and to supplement later histology. Others may be fixed later and stored frozen for possible cytochemistry and immunocytochemistry for cell surface and other antigens or interphase FISH.[50]

Needle core biopsy specimens: Touch imprint preparations are not generally used, but may be of use in the rare cases an FNA has not been performed. The tissue used for imprint can then be used for FCM studies. A separate core of undamaged tissue should be submitted for formalin fixation and paraffin embedding.

Bone marrow specimens: Trephine touch imprints are desirable for morphology, cytochemistry, and immunohistochemistry, especially if an adequate aspirate could not be obtained.[51]

Use of imprints for immunostaining: Fixation in 0.1% formol saline for 2–14 hours eliminates the troublesome background protein and red blood cells, and provides excellent preservation of lymphocyte membrane antigens other than immunoglobulin.[52]

iii. Fix sufficient tissue for good histology

Well-prepared, formalin-fixed, paraffin-embedded sections remain the gold standard for lymph node diagnosis and are the highest priority of triage.

Guideline — Lymph node diagnosis — 'gold standard'	Level of evidence	Refs
Well-prepared, formalin-fixed, paraffin-embedded sections remain the gold standard for lymph node diagnosis and are the highest priority of triage.	IV	53

Cut a number of 2 mm slices of lymphoid tissue and place immediately in 5–10 times their volume of fixative for morphologic diagnosis. Immunohistochemistry, molecular studies (by PCR or FISH) can also be performed on this tissue.

iv. Submit a cell suspension for flow cytometric analysis

For FCM analysis, all specimens must be in a single cell suspension. Unless the specimen can be delivered to the flow laboratory immediately, the specimen is usually suspended in RPMI 1640 medium stored at 4°C, Hanks solution, or physiological saline. This specimen should reach the laboratory within 24 hours, but useful results can sometimes be obtained with an even longer delay. Suspected cases of Burkitt's lymphoma/leukaemia and cerebrospinal fluid require more rapid transport and processing. It is recommended that if the specimens are received for immunophenotyping 24 hours or more after collection, a viability test, for example, trypan blue exclusion test, is performed.

FNA: The aspirate is simply expelled into the appropriate fluid and transported at 4°C or room temperature.

Cytological specimens other than FNA: Specimens may be submitted directly to the flow laboratory if a delay of less than two hours can be ensured. For longer delays, the specimens should be placed in RPMI 1640 or Hanks solution and transported at 4°C or room temperature. There is no need for anti-coagulation of cytological specimens such as pleural, ascitic or cerebrospinal fluid.

Surgical or NCB: A thin slice of lymphoma tissue should be placed in RPMI 1640 at 4°C. It is important that it be transported at this temperature to slow autolysis. For lymph nodes, a very effective alternative technique is to scrape a scalpel blade over the cut surface of freshly sliced tissue — usually taken from one end of the node to preserve the central slices for histological section.[54] The scrapings are then placed in RPMI 1640 as above.

Blood or bone marrow: The blood or bone marrow aspirate should be collected and placed in an anticoagulant, either EDTA or heparin, and transported as above.

Immunophenotyping of every case is not universally recommended. Generally it is carried out in patients in whom the morphology is inconclusive or the lymphoma needs to be subtyped.

Additional allocations depending on the indications and the amount of tissue available

i Sterile specimen for microbiology. Transport at 4°C

ii Specimen frozen for intraoperative assessment (see Section 4.3.3)

iii Other ancillary techniques.

The majority of lymphomas can be diagnosed and classified by morphology plus immunophenotyping for lineage and clonality, using a combination of immunohistochemistry and flow cytometry. Only a minority of cases require molecular genetic testing or cytogenetics.[55–58] Increasingly, these tests can be done on formalin-fixed, paraffin embedded tissues. The method used depends on the differential diagnosis.[55–61] RNA detection methods still require fresh or frozen material[57], and many institutions cryopreserve fresh tissue for possible future studies using emerging technology such as microarrays. However, for small specimens, a good fixed sample for morphology always takes precedence.[45]

a Frozen tissue (refer also to Section 4.3.3):

- i Immunostains for cell surface and other antigens that do not survive paraffin processing (infrequently required)
- ii Molecular studies by Southern blot, PCR, and techniques where RNA is needed
- iii Cryostorage for:
  - a Emerging techniques such as cDNA microarrays
  - b Tissue banking for clinical trials and laboratory research (subject to ethics approval and patient consent)

b Sterile specimen in RPMI or physiological saline (5x2x2 mm) for:

- i Molecular studies. (Unless there is an immediate indication for these techniques, freezing tissue is more economical) (refer to Section 4.3.3).>>>
- ii Metaphase cytogenetics and FISH techniques in RPMI tissue culture medium or preferably, immediate delivery at room temperature, or at 4°C if more than two hours delay (see Chapter 6).

c Electron microscopy. Small blocks of tissue <1 mm thick in 2.5% glutaraldehyde kept at 4°C for electron microscopy, especially if intraoperative microscopy shows large anaplastic malignant cells. (Note that well-fixed tissue can also be retrieved from neutral buffered formalin for electron microscopy).

4.3.3 Freezing tissue

Tissue to be frozen for intraoperative diagnosis and later ancillary tests should be placed in optical cutting temperature (OCT) embedding compound on a cryostat chuck, and snap frozen in a super-cooled mixture such as isopentane and dry ice mixture (–79°C). Freezing of slices less than 1–2 mm thick on a rapid-freeze chuck accessory in a cryotome can be satisfactory, though not ideal. Freezing in liquid nitrogen (<–195°C), or in an EM embedding capsule in liquid nitrogen and isopentane (–150°C) are preferable if intraoperative frozen section is not required.[1,45–47,57,62] Frozen sections can also be transported.

For storage, frozen tissue should be wrapped in aluminium foil or plastic to avoid desiccation. Storage is ideally at –70 or –80°C, especially for nucleic acids, but –20°C is sufficient for many antigens. Any thawing must be avoided and tissue should not be stored in cryostats or freezers with automatic defrost cycles. Frozen tissue should be transported in a Styrofoam container with sufficient dry ice to prevent thawing in transit. Long-term cryopreservation should be at <170°C.

4.3.4 Fixation of tissue

Ten per cent neutral buffered formalin is the recommended general fixative for lymphoma diagnosis. It is universally available, inexpensive, stable and relatively safe. Fixation time is not critical and it

can be disposed of without environmental problems. It can be used in tissue processors and autostainers with other routine tissue. Preservation of antigens (using antigen retrieval) is good if adequately fixed. Fixation should not exceed 24 hours.[63,64] Fixation in 10% neutral buffered formalin also ensures that DNA is well preserved for PCR studies.[45,47,51,57,65,66]

For bone marrow biopsies, addition of acetic acid (formol acetic acid) has been reported to improve cytology.[63]

Laboratories with a special interest in haematopathology may use other fixatives. Metal-based fixatives such as B5 and Zenker's, and acid fixatives such as Bouin's, give good morphology and immunohistochemistry, but require special preparation and processing. Mercury is a toxic environmental pollutant that is difficult to dispose of safely, and picric acid is toxic and explosive. B5 is commonly used.[1,44] Mercuric fixatives and Bouin's are not recommended for PCR[65,67], though satisfactory results have been reported with B5.[68] Routine use of these fixatives has diminished due to environmental concerns.

4.3.5 Decalcification of tissue

Bone marrow trephines are routinely decalcified before processing. Other specimens involving bone may also require decalcification.

Decalcification with 10% neutral EDTA is superior to stronger acid decalcifiers such as RDO for immunohistochemstry.[63] Formic acid decalcification is also satisfactory for immunohistochemistry[64], but has been reported as inferior to EDTA for PCR.[69] Decalcifying agents containing hydrochloric acid, such as RDO, are best avoided because they produce the carcinogenic by-product bis-chloromethyl ether when mixed with formaldehyde.[70–72] Hydrochloric and picric acids damage antigenicity and should be avoided.

4.4 Referral of lymphoma material

4.4.1 General note

Centres performing biopsies for diagnosis of lymphoma need to develop referral arrangements for ancillary studies not available locally. Access to immunohistochemistry and microbiology services is necessary, and routine availability of flow cytometry is strongly recommended.[1] Molecular genetics techniques should be available in selected cases.

4.4.2 Referral of fresh, frozen or fixed tissue or cells for ancillary studies

See transportation requirements described in previous section

4.4.3 Referral of processed lymphoma material for histologic second opinion or review prior to therapy

Second opinion may be requested by an anatomical pathologist because of diagnostic difficulty, or by a treating clinician prior to therapy. To minimise delays and waste of tissue, it is often convenient to refer the material to the pathologist who functions as a member of the multidisciplinary team where the patient will be treated.

The consulting pathologist must have access to haematoxylin and eosin stained slides, immunohistochemistry, the original pathologist's report, clinical details, and results of any ancillary studies such as flow cytometry and molecular genetics. Ideally, the original slides including H&E and immunohistochemistry stains should be sent (to be returned to the original pathologist after review).

Either a representative paraffin block (to be returned) or at least 12 unstained sections on sialanised or charged slides (to be retained by the consultant).

At present, there is no Medicare funding to cover referral for second opinion. Strategies for funding referrals must be addressed so that cost does not act as a disincentive.[73]

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