

Field Workers' Forum

BIOPSIES IN LEPROSY

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Principles and Usefulness

Leprosy presents a considerable problem to doctors in many parts of the world. Not only are cases of the disease to be found in most countries nowadays, but as knowledge of leprosy grows, so also do we come to appreciate more fully the difficulties in assessing accurately the immunological status of each case, in deciding the most suitable therapy, and in carrying it through to a successful conclusion. Throughout the course of the disease the taking and reading of biopsies from patients can be a valuable aid to diagnosis, classification, the assessment of progress under therapy, and also in deciding when the infection may be regarded as completely subsided. The biopsy in leprosy may be compared with X-ray examination in tuberculosis. Although it is possible to deal with tuberculosis without using X-rays, their value in diagnosis and in the care of patients is unquestioned. The same applies where biopsies are concerned to the prolonged and quiet infection with the leprosy bacillus. Their usefulness may be considered under four heads, 1. Diagnosis; 2. Classification; 3. Progress; 4. Subsidence.

1. DIAGNOSIS

If the leprosy problem in the world is to be faced adequately, early diagnosis is essential. This may be difficult clinically, especially in patients presenting with early leprosy, because at this stage the disease is often easily confused with other dermatological conditions. In its earliest stages lepromatous leprosy may be totally lacking in distinctive signs, so that clinically it is impossible to tell that the patient has leprosy. By the time skin lesions become visible even to the trained eye, the infection is already well established, particularly in neurovascular tissue and perhaps plain muscle. Before this stage is reached, a skin smear, depending on where it is taken, could at best show only a few bacilli, and so could provide only inconclusive evidence of leprosy type and extent. A biopsy correctly taken could make everything plain.

In patients whose leprosy is in the borderline (BB) to borderline lepromatous (BL) area of the spectrum, the infection may sometimes be largely confined to neural tissue for many months, with no obvious skin lesions. The immunological response will however give rise to signs and symptoms of nerve involvement such as slight enlargement and tenderness, with subjective feelings of tingling, burning, pins and needles, formication, heaviness etc. in the area of supply. In such cases, if leprosy is suspected, a biopsy from an affected cutaneous branch will establish the diagnosis.

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In types of leprosy towards the tuberculoid end of the spectrum the patient may present with either an actual visible skin lesion or else an area of skin with signs of sensory impairment. Here, skin smears are useless for diagnostic purposes, because by the time the patient presents himself with such lesions such bacilli as were originally present will mostly have been destroyed. Biopsy will reveal the characteristic histopathology when the clinical diagnosis is in doubt.

2. CLASSIFICATION

The clinical manifestations of leprosy depend upon the immunological response of the patient to the presence of the *Myco. leprae*. This may range from an effective cell-mediated immunity to the complete lack of such immunity. It is important to assess the degree of defence as accurately as possible, as the care and treatment of the patient, and indeed the regulation of his future life in many of its aspects may depend on this. One can get some idea of the patient's position in the leprosy spectrum from the clinical examination, but it is only by seeing the actual cells involved in the immunological response and their reaction to the invading organisms that a really accurate assessment can be made at any given time. Biopsy makes this possible.

3. PROGRESS

If the patient has lepromatous leprosy, his cure depends on the bacteriostatic and/or bacteriocidal activity of drugs. While routine skin smears offer a rough and ready means of assessing progress under therapy, biopsies, repeated from time to time, provide the most refined means of doing this. Biopsy will reveal bacilli lying in the more protected sites, in Schwann cells, muscle cells, hair follicle and blood vessel wall cells not easily sampled by the slit-smear technique.

If treatment has been irregular or insufficient, or if drug resistance is developing, viable organisms in these more protected areas will multiply and disseminate. An impending relapse can generally be detected earlier by means of biopsy than by skin smear. It can certainly be diagnosed much earlier by biopsy than by clinical manifestations. A biopsy taken every 6 to 9 months after apparent quiescence in a lepromatous patient can thus be a valuable safeguard.

Where there is some cell-mediated immunological response, that is in the borderline lepromatous (BL) to tuberculoid (TT) range of the spectrum, the effectiveness of the killing and digesting action of the histiocytes on the organisms can be monitored by means of periodic biopsies. The nearer to the TT side, the more effective is the digestive process and the quicker the healing, and there is no point in taking biopsies when erythema and infiltration have subsided clinically, though any point of persistent inflammation merits attention. The nearer to the BL side, the greater is the risk of interference in any healing process, and regular supervision by means of biopsy is valuable.

During the course of leprosy infection, more acute inflammatory changes may occur in the skin whether as a result of an altered immunological response to *Myco. leprae* or to other unrelated conditions. Here again a biopsy may be valuable in determining the cause.

4. SUBSIDENCE

Complete subsidence of infection in the borderline tuberculoid (BT) to tuberculoid (TT) range of cases is generally visible, or, in the case of nerves, palpable clinically. With the BL to BB area of the spectrum, where there has been

a greater dissemination of organisms, it is not easy to ascertain whether infection persists, in neural tissue in particular. Here again, biopsies taken from cutaneous nerve branches may be of some assistance.

With lepromatous patients careful follow up is essential, even when skin smears are negative. It is impossible to investigate all the sites where viable bacilli may persist, but some are available to biopsy, e.g. hair-bearing skin from the facial region or limbs, concentrating upon arrector pili muscles and hair follicle cells and blood vessel walls; cutaneous nerve branches; the plain muscle in the scrotal skin in the male; the plain muscle in the nipple region in both male and female.

Practical Notes on the Taking of Biopsies in Leprosy

Leprosy manifests itself mainly in the superficial tissues of the body. For diagnostic and classification purposes biopsies are taken from skin and/or cutaneous nerves.

It is important always to explain the necessity for a biopsy to the patient and to enlist his co-operation, especially as follow up biopsies may be required. To this end it is essential that the operation should be performed as carefully and as painlessly as possible. Sometimes a suitable biopsy can be taken without trouble to the patient during the performance of some other operative procedure.

The whole biopsy should always be taken from pathological tissue and should not be wasted on surrounding normal tissue, which is of no use at all for diagnostic or classification purposes.

A. SKIN

1. The number and distribution of skin lesions will depend upon the present position of the patient in the leprosy spectrum. When there is a choice always take biopsies from lesions of the face or from lesions on the external aspects of limbs. These areas provide better living conditions for the bacilli than anywhere on the trunk, and therefore a more accurate pathological picture will be obtained.

There may be misgivings about the taking of facial biopsies, but it should be remembered that they do not inconvenience the patient appreciably and that they heal quickly and well.

2. Always take the biopsy from the most active part of the lesion—that part showing erythema, infiltration etc. This is usually the margin in cases where there is some degree of inflammatory response to the presence of the *Myco. leprae*.

3. Where necessary, for accurate initial diagnosis and assessment, take biopsies from two or more lesions in different parts of the body. In this regard it should be remembered that because the face provides good living conditions for the *Myco. leprae*, the pathology tends to be a little more towards the lepromatous side of the spectrum in comparison with biopsies from other parts of the body.

4. Always use adequate local anaesthesia when excising a tissue. Inject the anaesthetic fluid into the subcutaneous tissue under the selected area and wait for it to take.

5. Always exercise great care to prevent damage to the tissues. Instruments should be sharp and fine—eye instruments are excellent. The tissue should be handled as little as possible and only picked up gently by forceps at one end. Do NOT transfix the tissue with a suture.

6. Always take a biopsy of adequate size. For general purposes this should be

elliptical, 1.5 cm long by 0.5 cm wide. *It must be full thickness*, the incision extending vertically downwards to include subcutaneous fat.

Under some working conditions it is not possible to use a knife for excision. Biopsy punches may be used but the drawbacks must be recognized *viz.* a smaller specimen, more likelihood of damage to the tissues, more difficulty in stitching the wound, difficulty in keeping the punches sharp etc.

7. Always stitch the wound carefully and neatly so as to get satisfactory healing. Apply an occlusive plaster dressing. Advise against excessive movement of the part for a day or two. If necessary give an analgesic for post-operative pain. Remove stitches in 4-5 days (face) or 7 days (other parts of the body).

B. CUTANEOUS NERVE

A more accurate assessment of the pathological state of the patient is generally obtained from nerve biopsy than from skin. If a cutaneous nerve is clinically enlarged and/or tender it will already show destruction of most of its fibres, so there is no harm in taking biopsies from it.

Even if the biopsy should consist of normal nerve, the resultant area of sensory loss is only small and is generally only temporary, as there is "growing in" from neighbouring cutaneous nerves.

The biopsy procedure is the same as that for skin, though a longer incision may be necessary for adequate exposure. If possible a piece of nerve 1.5 cm in length is removed. This may be the whole thickness of the nerve if it is smallish, or a segment of the nerve if it is much enlarged. A small hook is useful for picking up and clearing the nerve from surrounding tissues before excision.

When fixed, the nerve can be cut by the technician into two pieces, 1.0 cm and 0.5 cm in length, and these can then be blocked together, one placed horizontally and the other vertically. On section a better appreciation of the extent of involvement of the various bundles is then obtained.

C. CHECKING FOR RESIDUAL BACILLARY ACTIVITY

When checking on lepromatous or near lepromatous cases that have shown response to treatment and in which the Bacillary Index is 0.0, it is advisable to take biopsies that will include bacillary "hide-outs". These are nerve and plain muscle, and possibly hair follicle cells. Therefore cutaneous nerve bundles should be sampled, and skin biopsies should be from hair-bearing areas on the limbs and, if possible, in the male from scrotal skin. The biopsies should all be full thickness so as to include larger blood vessels. In this way hair follicles, arrector pili muscle, dartos muscle and the muscle walls of blood vessels will be examined.

Fixation of Leprosy Tissues

The most satisfactory fixative for leprosy tissues is Formol Zenker (Lowy, 1956). The formula for this is:—

A	Formaldehyde solution (40%)	10 ml
	Mercuric chloride	2 g
	Distilled water	90 ml
B	Glacial acetic acid	

A may be made up and kept in a stock bottle according to requirements. It should be kept in a cool place.

B must be added to *A* just before use at the time of biopsy.

- Note.* (1) Sometimes there is difficulty in obtaining mercuric chloride from local sources, but it can usually be obtained from agents of large pharmaceutical firms.
- (2) On making up the stock solution *A* it is best to dissolve the mercuric chloride first in the formaldehyde. This may take an hour or two and require occasional stirring and shaking. The distilled water may then be added. Sometimes solution of the mercuric chloride is not absolutely complete and there is some deposit in the bottle. This is of no consequence.

The amount of fixative fluid should be 20 times the volume of the tissue. For the average biopsy (1.5 cm × 0.5 cm × full thickness) 20 ml is adequate and this is obtained by adding 1 ml of *B* to 19 ml of *A*.

After excision excess blood should be cleaned off the tissue, which is then placed upon a small piece of filter paper so that it sticks to it and does not curl up. This is then immediately placed in the fixative solution for a period of not less than 15 h and not more than 24 h i.e. until the next day. The fixative is then poured off and is replaced by 70% alcohol. The tissue is then ready for processing locally, if this is possible, or it may be despatched in the alcohol for processing.

70% alcohol is made up of absolute alcohol 70 parts and distilled water 30 parts. If absolute alcohol for dilution is not obtainable, then methylated spirit (undiluted) may be used.

If a large biopsy tissue is excised then 1. increase the amount of fixative fluid accordingly (20 times the volume of the tissue) and 2. score the tissue with a sharp knife in parallel lines 0.5 cm apart so as to facilitate penetration of the fixative fluid.

Despatch of Biopsy Material after Fixation

If there is only one biopsy to be sent it may stay in its bottle in the alcohol. Put the identifying name and/or number on the outside of the bottle and also the name and address of the sender.

If there are several biopsies to be sent the most convenient and economical way is to wrap up each biopsy tissue separately with its identifying name and/or number and send them all together in one bottle. For this purpose cut pieces of gauze 5 cm square. Also pieces of stiff paper or post card 1 × 2 cm. Write the identifying name and/or number of the first biopsy tissue on one side of a piece of the paper with a *soft lead pencil* and place it with the appropriate specimen on a square of gauze. The corners of the gauze are then brought together and tied with cotton to make a loose little bundle. Place this bundle immediately into 70% alcohol in a suitably sized bottle with a plastic screw top or rubber stopper. Do *not* use ink or ball point for writing on the paper as these will wash out in the alcohol. Proceed in the same way with each specimen. Several biopsies may thus be sent together in the one small bottle. Only sufficient alcohol is required to keep the gauze-wrapped tissues wet. Screw down or insert the bottle top firmly, and if necessary wax it or fix it with adhesive tape. Put the name and address of the sender on the outside of the bottle. Wrap the bottle around with some corrugated cardboard to protect against damage. Enclose a letter with the clinical

information (see below) and complete the package according to postal regulations. For Customs purposes state "Pathological specimens. No value".

Note. Metal bottle tops are corroded if there is any contamination of them by the mercuric chloride. Always use plastic tops if possible.

NEVER use rubber glove fingers as the container for the biopsy as they frequently leak, and the tissue is dried out on arrival.

If mercuric chloride is not available, then use Formol saline as the fixative. The formula is as follows:—

Sodium chloride	0.9 g
Formaldehyde solution (40%)	10 ml
Distilled water	90 ml

The biopsy may be sent in this fixative fluid. Changing into 70% alcohol is not necessary.

This fixation method will result in some shrinkage of the tissues and in poorer staining quality as compared with the recommended Formol Zenker method.

Clinical Information

In order to assist accurate assessment of the biopsy specimens, it is necessary for the pathologist to have the relevant clinical information. If photographs of the lesions at the time of biopsy have been taken, these can be of great help. They may be sent on later after processing and printing.

The type of clinical information required is as follows:—

<i>Name</i>	<i>Sex</i>	<i>Age</i>	<i>Race</i>
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Hospital No. and/or Biopsy No.

History

- (1) Family history as regards leprosy.
- (2) Brief history of development of leprosy in patient—first signs or symptoms and subsequent spread.

On examination

- (1) Description of present lesions:—

Approximate number and distribution.

Are the lesions hypopigmented or hyperpigmented.

Are the lesions erythematous.

Are the lesions flat or raised.

Are the edges of the lesions vague or well defined.

Is there sensory loss in the lesions to cotton wool touch, to pin prick and to hot and cold testing.

- (2) Is there enlargement and/or tenderness of any of the peripheral nerves
 - a. nerve trunks.
 - b. cutaneous branches.

If so, give details of the nerves involved.

- (3) Is there any muscular wasting, weakness or paralysis. If so, give details of the muscles involved.

Therapy

- (1) Previous leprosy therapy.
- (2) Present leprosy therapy. Always give dosage in g, mg etc.

Laboratory examinations

- (1) Bacillary Index and Morphological Index. Dates when taken.
- (2) Other relevant laboratory findings.

*Clinical diagnosis**Biopsy*

- (1) Biopsy site.
- (2) Date when biopsy performed.
- (3) Fixative used if other than that recommended.

Photographs

- (1) Black and white.
- (2) Colour.

Processing and Staining

The following notes are added for the sake of any doctor who may be undertaking his own histopathological work.

A. DEHYDRATION

The amount of fluids to be used must be 10 times the volume of the tissues to be processed.

Durable use of absolute alcohol—40 specimens of skin may be passed through each 100 ml of fluid before discarding the fluid.

To dehydrate the tissue pass it through varying strengths of alcohols in the following way. The times given will suffice most skin specimens with the exception of tiny fragments or the larger tumours.

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|-----|--|------|
| (1) | 70% alcohol containing 0.5% iodine for | 2½ h |
| (2) | Absolute industrial spirit for | 1½ h |
| (3) | Absolute industrial spirit for | 1½ h |
| (4) | Absolute industrial spirit for | 1½ h |
| (5) | Absolute ethyl alcohol for | 2 h |

B. CLEARING

Durable use of the clearing reagent—100 ml may be used daily for up to three weeks.

Use either cedar wood oil (technical) for 12 to 18 h (overnight) or, if for leprosy investigation, pure turpentine oil may be used for 6 h only.

C. EMBEDDING

1. *Impregnation with paraffin wax.* Change the specimens from the clearing oil into an embedding bath of paraffin wax which has a melting point of 56°C. After 3 h change them into a second clean bath of paraffin wax of melting point 56°C. Allow to impregnate for a further 6 h.

2. *"Blocking" or moulding out.* Use "L" shaped moulding irons and clean metal base plates. Prepare fresh paper-filtered wax which has a melting point of 58° to 60°C to the required amount, preferably Fibrowax. Pour into the mould and place the tissue to the bottom of the wax on its side. Allow to cool at room temperature.

If the specimen is a nerve and is of adequate length, cut it into two pieces and block them together so as to obtain longitudinal and transverse sections.

D. PREPARATION FOR CUTTING

Chop up the cooled, hardened paraffin blocks, mount on chucks and allow to cool off again before trimming. Trim off the superfluous wax covering the under surface of the tissue and continue trimming until a complete cross section of the specimen has been exposed.

Microtomes. With regard to microtomes and knives nothing can be gained by suggesting one kind or another, as this is purely a personal matter. Experience shows that the best tools are those which the worker has become accustomed to using.

Knives. Either wedge-shaped or biconcave knives may be used for cutting skin. Wedge knives are used on a base sledge microtome, while biconcave knives are best used on rotary or Cambridge rocker pattern microtomes.

Cutting sections. The quality of the section cutting will depend entirely upon the standard of the knife sharpening technique. This is not good enough if, in the act of cutting, the block will not pass over the blade slowly, and if clean, flat sections are not obtained. If sections are cut by "chopping" the block over the knife edge, bad creasing and uneven staining will result. Skin tissue should always be cut epidermis first. After trimming the paraffin blocks, place them face down on a tray of ice for 20 min.

Sections are then cut at 4 to 5 μ in ribbon formation. To each spell of icing cut only one ribbon of 24 sections. Float the cut sections on to a cold water bath for safety while further sections are being cut. Next divide the ribbons into short strips of 4 to 5 sections and lift off and transfer to a warm bath of water (45°C) by using a clean slide and a dissecting needle. Allow them to flatten for a few seconds, then mount them on to glass slides, previously cleaned in methylated spirit, and which have been faced with a thin smear of egg albumen to allow adhesion of the section to the slide.

Glycerin albumen

White of an egg	1 volume
Glycerol	1 volume
Distilled water	1 volume

Mix well by shaking intermittently and then add a few crystals of thymol as a bacteriocide.

The slides are numbered with a glass diamond marker and dried on a hot plate at 50°C for 2 h, or preferably left in an incubator at 37°C for 24 h, when they are ready for staining.

Investigation of tissue for leprosy or other acid-fast organisms. It will be noted that iodine in 70% alcohol has been used in the process of dehydration and that pure turpentine oil has been used as the clearing agent. In view of this it will not now be necessary to treat the paraffin sections for mercury deposits, if Formol Zenker fixation has been used. Neither will it be necessary to impregnate the sections with turpentine oil before staining for organisms.

Staining of Leprosy Sections

A combination of Masson's trichrome stain (for the tissues) and the Fite-Faraco modification of the Ziehl-Neelsen stain (for the bacilli) is recommended. This has

been given the name of the TRIFF staining method from TRI for trichrome and FF for Fite-Faraco (Wheeler *et al.*, 1965). This shows very clearly the interaction between defence cells and *Myco. leprae*. It also has the added attractions of economy in time of staining and in materials, in storage space for the stained slides, and provides a good picture for photographic purposes. The details of the method are as follows:—

1. Warm slide gently to melt wax.
2. Place in pure turpentine oil for 30 min.
3. Blot gently with filter paper, wash in tap water for 3-5 min and place in common solution of iodine (0.5% iodine crystals in 70% alcohol) for 5 min to remove mercuric salts.
4. Rinse thoroughly with tap water.
5. Immerse in 1% aqueous solution of sodium thiosulphate until bleached, approximately 10-20 s.
6. Wash in running water for 5 min.
7. Stain with cold strong filtered carbol fuchsin (Ziehl-Neelsen) for 30 min.
8. Wash off surplus stain with tap water for 5 min.
9. Decolourize with Fite decolourizer (0.5% hydrochloric acid in 70% alcohol) for 20 s. Then dip into absolute alcohol for a few seconds.
10. Wash thoroughly with tap water for 15 min.
11. Stain with Harris's haematoxylin for 2-3 min.
12. Wash in tap water until blue—approximately 5 min.
13. Rinse rapidly—for a few seconds only—in Fite decolourizer.
14. Wash for at least 10 min with tap water.
15. Stain with 1% aqueous yellow eosin for 15 s.
16. Wash thoroughly with tap water for 10 s. Change slides into a clean rack. Immerse in 90% alcohol for 10 s.
17. Rinse over rapidly with absolute alcohol, in two different containers, for a few seconds in each one.
18. Stain with 2% alcoholic solution of saffron for 5-15 min (roughly 7 min but control with the microscope to ensure differential staining of nerves and connective tissue, a yellow colour). Rinse in alcohol to remove excess saffron and replace in the stain if not stained sufficiently.
19. Rinse rapidly with absolute alcohol, in two different containers, for a few seconds in each one.
20. Clear with xylene.
21. Mount under a coverslip in DePeX.

N.B. If iodine in alcohol has been used in dehydration and the turpentine oil in clearing, then the early stages of the TRIFF staining are:—

1. Warm slide gently to melt wax.
2. Place in pure turpentine oil for 1 min to remove wax.
3. Blot gently with filter paper and wash in tap water.

Then proceed with stage 7—staining with carbol fuchsin.

The staining scheme is:—

Nuclei	Blue
Cytoplasm	Pink
Muscle	Pink
Myelin	Pink

Collagen	Yellow
Acid-fast organisms	Red

2% alcoholic solution of saffron may be obtained from:—

Raymond A. Lamb,
6 Sunbeam Road,
London, NW10 6JL.

In the past, outside fully developed countries, the use of biopsies in leprology has been confined mainly to research centres and specialized units. As leprosy becomes increasingly integrated into Health Services, and as laboratory facilities become more sophisticated and more widely available, there can be no doubt that much greater emphasis should be placed on biopsies, both in the best interests of patients and the efficient practice of leprology. As an aid to diagnosis and effective follow through in many patients, histopathology is of great and increasing value, and deserves to become a routine procedure wherever efficient laboratory work is not hampered by economic strictures.

Most leprosy biopsies consist of skin, and this is not an easy tissue to deal with. Some technical training is necessary, and the opportunity for technicians to obtain this in some laboratory dealing with dermatology should seriously be considered. In the meantime it is possible to send biopsies for processing to a few laboratories and obtain an expert report and a stained section. The Leprosy Study Centre, London (Address: 57a Wimpole Street, London W1M 7DF) is very willing to assist in this way and also provides facilities for laboratory technicians to practise processing, cutting and staining of leprosy tissues.

Finally, it is only as we gradually accumulate and study pathological material from leprosy patients—and this means biopsy and post mortem material from all parts of the body—that a clearer picture will be obtained of the way in which the extremely mild organism *Mycob. leprae* lives and spreads in the body, and of the way in which the host's tissues attempt to deal with it. This is the foundation on which the leprosy eradication programme must ultimately be built.

References

- Lowy, L. (1956). *J. med. Lab. Technol.* **13**, 558.
Wheeler, E. A. *et al.* (1965). *Lepr. Rev.* **36**, 37.