Correlation of quantitative tests of nerve and target organ dysfunction with skin immunohistology in leprosy

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Summary
Loss of nociception and hypohidrosis in skin are hallmarks of leprosy, attributed to early invasion by Mycobacterium leprae of Schwann cells related to unmyelinated nerve fibres. We have studied skin lesions and contralateral clinically unaffected skin in 28 patients across the leprosy spectrum with a range of selective quantitative sensory and autonomic tests, prior to biopsy of both sites. Unaffected sites showed normal skin innervation, when antibodies to the pan-neuronal marker PGP (protein gene product) 9.5 were used, with the exception of intra-epidermal fibres which were not detected in the majority of cases. Elevation of thermal thresholds and reduced sensory axon-reflex flare responses in affected skin correlated with decreased nerve fibres in the sub-epidermis, e.g. axon-reflex flux units (means ± SEM) for no detectable innervation; decreased innervation; and clinically unaffected skin, were 23 ± 3.1; 41.2 ± 7.3; and 84.5 ± 4.0, respectively. Reduced nicotine-induced axon-reflex sweating was correlated with decreased innervation of sweat glands. Where methacholine-induced direct activation of sweat glands was affected, there was inflammatory infiltrate and loss of sweat gland structure. This study demonstrates a correlation between selective nerve dysfunction on clinical tests and morphological changes in skin, irrespective of the type of leprosy, and is the first to show that loss of sweating in leprosy may result either from decreased innervation and/or involvement of the sweat glands. The findings have implications for the selection and monitoring of patients with leprosy in clinical trials which aim to restore cutaneous function.

Keywords: leprosy; immunohistochemistry; skin

Introduction
Leprosy is one of the most common diseases of peripheral nerves world-wide. It is caused by infection with Mycobacterium leprae, discovered by Hansen in 1873. Depending on their immunological status, patients may develop disease ranging from the tuberculoid to the lepromatous type (Ridley and Jopling, 1966). The former occurs in those with a degree of immunity, and may be clinically confined to hypopigmented skin patches, with or without evidence of neuritis, while the latter is more diffuse. The clinical features combined with skin histology provide a definitive diagnosis along the immunological spectrum. The involved nerves are often thickened. Although antibacterial drugs are effective, failure of nerve regeneration and trauma leads to trophic ulceration.

There is early involvement of the cutaneous innervation in all types of leprosy (Khanolkar, 1952), with degrees of impairment of pain and temperature sensation, axon-reflex flare responses and sweating. In accord, the earliest reported nerve lesions in human leprosy and animal models are in unmyelinated fibres and their Schwann cells (see Shetty et al., 1988). Our previous work and that of colleagues has provided evidence that unmyelinated afferents containing substance P and nerve growth factor, on which they are dependent, are implicated in leprous neuropathy (Anand et al., 1983a; also see Anand, 1996, 1997).

The skin lesions in leprosy are accessible and may be relatively well circumscribed, providing an opportunity to study the role of small sensory and autonomic fibres and trophic changes in human neuropathy. However, quantitation of function of fine calibre cutaneous nerves has been difficult, as has their identification in skin biopsies. Our aim in the present study was to obtain skin biopsies from affected...
Table 1: Summary of patients’ characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
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<td>Diagnosis</td>
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<td>Male : female</td>
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*Unless indicated otherwise.

Methods

Patients

Leprosy patients (n = 28) were recruited at the Acworth and Bombay Hospitals, Bombay, India across the entire range of the disease spectrum. Ethical permission for the study was given by the Ethics Committee of the Bombay Hospital Medical Research Centre, Bombay, and informed consent from patients was obtained prior to clinical tests and skin biopsy. Most patients (19 out of 28) had untreated disease, whilst others had received treatment (multi-drug therapy) for up to 26 months prior to the study. The patients were diagnosed clinically and classified within the leprosy spectrum, following appropriate electrophysiological and pathological investigations. The patients selected had circumscribed lesions, with no clinical evidence of a lesion on the contralateral mirror-image site (the latter was difficult with polar lepromatous cases). The numbers of patients, diagnoses and test regions are shown in Table 1.

Both affected and contralateral, clinically unaffected, sites were studied with quantitative sensory and autonomic function tests, which have been described fully in previous publications (Anand, 1992, Anand et al., 1983b, 1996). They are described in brief below.

Thermal threshold

A thermal threshold testing system with a rate of rise in temperature of 1°C/s was used. The thermode was placed at the appropriate affected or unaffected site prior to biopsy. The baseline temperature was set at a neutral point, between 30 and 32°C. Thermal thresholds were determined for cool and warm sensation, and for heat perceived as pain. Four separate consecutive tests were carried out for each modality. The mean difference from the baseline temperature was recorded as the threshold.

Light touch threshold

Light touch thresholds were determined using Semmes-Weinstein hairs placed on the skin by slightly bending the hair. The number of the hair with the lowest strength felt by the patient was recorded. Values were then converted into the respective gram value.

Sweating studies

Directly stimulated sweating, which requires the presence of functioning sweat glands, was induced with intradermal methacholine (4 µg in 0.1 ml H2O), and axon-reflex sweating, which is dependent on the function of local sympathetic nerve fibres, with intradermal nicotine (0.8 µg in 0.2 ml H2O) (see Abdel-Rahman et al., 1992; Anand et al., 1996). Sweating was measured with an evaporimeter (Servomed, Stockholm) in g/m²/h.

Axon-reflex vasodilatation

Sensory axon-reflex vasodilatation was determined by measurement of cutaneous blood flow using laser Doppler fluxmetry after injection of 0.01 ml of a 5 mg/ml capsaicin solution intradermally. The difference between the basal blood flow and the maximal blood flow after injection was recorded in flux units.

Immunohistochemistry

Full thickness, elliptical skin biopsies were obtained from tested sites under local anaesthetic. The biopsies were immersed in Zamboni’s fixative (2% w/v formalin; 0.1 M phosphate; 15% v/v saturated picric acid) for 4–6 h then washed by transferring to PBS (0.1 M phosphate; 0.9% w/v saline; pH 7.3) containing 15% w/v sucrose and 0.01% w/v sodium azide. After washing, the tissue was instantly frozen in melting isopentane and frozen sections (5–10 µm) collected on poly-L-lysine-coated microscope slides for processing with haematoxylin and eosin or immunostaining as described previously (Bar et al., 1997). Briefly, after blocking steps, tissue sections were incubated overnight with rabbit anti-PGP 9.5 (Ultraclone, UK) and sites of attachment of antibodies detected using an avidin–biotin peroxidase complex (Vector Labs, UK) and nickel-enhanced diaminobenzidine as chromogen (Shu et al., 1988). Negative control sections included incubation with normal rabbit serum instead of anti-PGP 9.5.

Preparations were counterstained in 1% w/v aqueous Neutral Red for nuclei and photographed using a Wild photomicroscope. PGP-immunoreactive nerve fibres in the epidermis, sub-epidermis or surrounding sweat glands at both sites were graded in three sections per biopsy as ‘abundant’
Skin innervation in leprosy

Fig. 1 Skin from clinically unaffected (A and C) and affected (B and D) sites from a patient with borderline tuberculoid leprosy showing normal epidermis (A) and sweat glands (C), and severe infiltration by inflammatory cells in affected skin, with eroded epidermis (B), and in sweat glands (D). Haematoxylin and eosin. Scale bar = 200 µm in A and B; 400 µm in C and D.

Results

Histology

All biopsies were full thickness and included hypodermal fat. Routine histological examination (haematoxylin and eosin) showed that epidermis and upper dermis from all clinically unaffected sites had a normal appearance, whilst biopsies from affected sites showed changes typical of the *M. leprae* infection. These included chronic inflammatory infiltrate, and in appropriate cases, extensive epithelioid granulomata with giant cells and, in some cases, epidermal erosion (Fig. 1A and B). In the lower dermis, sweat glands were often a target for inflammation in clinically affected sites, leading to glandular destruction and atrophy (Fig. 1C and D). Hallmark features of leprosy, including loss of dermal papillae and hypopigmentation, were seen in sections from affected sites (Fig. 2A and B). In one case there was clinical hyperpigmentation, and this was confirmed histologically.

Immunohistochemistry and clinical test correlations

Sensory tests

The clinically unaffected sites had normal responses to sensory tests, and showed ‘abundant’ distribution of PGP 9.5-immunoreactive nerve fibres in the sub-epidermal plexus. However, intra-epidermal nerve fibres were absent in 16 out of 28 cases (Fig. 2). Unaffected sites were indistinguishable from non-leprosy control skin for sensory tests and sub-epidermal innervation.
Clinically unaffected (A) and affected (B) skin from patients with borderline leprosy or tuberculoid disease, respectively, immunostained with antibodies to PGP 9.5. Typically, the affected site, although less severe than the case in Fig. 1, shows loss of intra-epidermal nerve fibres, flattening of dermal papillae and hypopigmentation. Immunoperoxidase with nuclear counterstain (Neutral Red). Scale bar = 100 μm.

In affected sites, biopsies which showed ‘absent’ sub-epidermal PGP 9.5-immunoreactive fibres (16 out of 28) had markedly elevated thermal thresholds, and showed severely reduced axon-reflex vasodilatation (Fig. 3). The vast majority of these sites were completely anaesthetic to cotton wool, pinprick and monofilament tests.

Biopsies from some patients (12 out of 28) showed some sub-epidermal PGP 9.5-immunoreactive nerves, which were ‘fewer’ than in the corresponding unaffected site and non-leprosy control skin. These affected sites showed partial preservation of function, and moderate abnormalities on thermal threshold and axon-reflex vasodilatation tests (Fig. 3).

Elevation of thermal thresholds and reduced axon-reflex flare responses in affected skin correlated with decreased nerve fibres in the sub-epidermis (Fig. 3A–C). Note that one patient declined the axon-reflex vasodilatation test on the unaffected side, and that in two other patients thermal thresholds were not performed, as the apparatus was not available. The axon-reflex flux units (mean ± SEM) were 23 ± 3.1; 41.2 ± 7.3; and 83.2 ± 2.7 for no detectable
Fig. 3 Scatter plots correlating unaffected and affected sites after capsaicin-induced axon-reflex vasodilatation (A), and warm (B) and cool (C) threshold-testing with degree of PGP-immunoreactive innervation. Boxed values correspond to the maximum and minimum temperatures applied (to remain within safe test limits).
Fig. 4 Correlation of sweat responses with eccrine gland and PGP-immunoreactive nerve morphology. Decreased axon-reflex or direct sweating correlated with decreased PGP-immunoreactive innervation or loss of gland, respectively. In cases where glands were preserved, despite loss of innervation, a good direct sweating response was obtained.

innervation; decreased innervation; and clinically unaffected skin, respectively; all were significantly different from each other (P < 0.0001, Student’s unpaired t test). The warm thresholds (mean ± SEM) were as follows: no detectable innervation—only 2 out of 17 patients had values below the safety limit (50°C); decreased innervation 7.4 ± 1.5°C (excluding the value above the safety limit), clinically unaffected skin, 3.3 ± 0.3°C; the latter two sets of values were significantly different from each other (P < 0.01). The cool sensory thresholds were: no detectable innervation 6.3 ± 0.9°C (eliminating the 5 out of 17 values below the cut-off limit of 20°C); decreased innervation 4.3 ± 0.6°C (eliminating the one value below the cut-off limit), clinically unaffected skin 2.2 ± 0.3°C; all significantly different from each other (P < 0.001). The absolute individual values for warm and cool sensory thresholds are shown in Fig. 3. The heat as pain thresholds were mostly above the safety limit in affected sites. In matched non-leprosy control subjects, for abdominal sites (leprosy n = 5, control subjects n = 6) axon-reflex flux units (mean ± SEM) were: clinically unaffected leprosy skin 89.2 ± 5.6, control subjects 82.8 ± 2.8; warm sensation, leprosy skin 1.5 ± 0.2°C, control subjects 2.0 ± 0.3°C.

Autonomic tests
The clinically unaffected sites had normal axon-reflex sweating (mean ± SEM) of 52.6 ± 1.8 g/m²/h, indicating an intact nerve and glandular function (Fig. 4). These showed ‘abundant’ PGP 9.5-immunoreactive nerves surrounding glands and ducts in the biopsy (Fig. 5A). These unaffected sites were similar to non-leprosy control subjects.

Some cases (5 out of 28) showed preservation of sweat gland morphology but lack of PGP 9.5-immunoreactive nerves in the region of the sweat glands (Fig. 5B). In these cases, a good response was obtained at the affected site from direct sweat gland stimulation, despite a very poor axon-reflex sweating response.

The majority (16 out of 28) of clinically affected sites showed inflammatory cell infiltration, loss of structure of sweat glands and loss of PGP 9.5-immunoreactive nerve fibres (Fig. 5C). These sites had very poor axon-reflex (6.5 ± 1.4 g/m²/h) and direct (8.7 ± 1.7 g/m²/h) sweating responses.

In some affected sites (7 out of 28), ‘fewer’ PGP 9.5-immunoreactive nerves supplied preserved sweat glands, and these had a significantly reduced axon-reflex sweating response compared with clinically unaffected skin (P < 0.002, Student’s unpaired t test), but a similar directly stimulated sweat gland response (P > 0.05) (Fig. 4).

No consistent relationship was found between the regional density of nerves and the immunopathological diagnosis in individual sites, or with prior multi-drug treatment. A similar proportion of patients at the tuberculoid end of the spectrum had no detectable sub-epidermal innervation in affected sites (68%), compared with lepromatous patients (62%).

Discussion
The skin lesions in leprosy provide an opportunity to assess the relationship of clinical methods for quantitative testing of sensory and autonomic nerve function, and of their target organs, with histological findings. Our results showed that selective tests correlated well with the loss of regional PGP 9.5-immunoreactive innervation. These findings are also important in the diagnosis and treatment of leprosy. First, the clinical tests correlated primarily with the regional distribution of nerves and the integrity of the target organ, rather than with the immunopathological diagnosis. Any generalization with respect to individuals or sites appeared invalid; there was differential loss of function and regional innervation in biopsies across the leprosy spectrum. Secondly, loss of integrity of the target organ (sweat glands) was responsible for the functional deficit in some sites, with implications for clinical trials; agents that may improve nerve function, such as nerve growth factors, would not be expected to improve sweating if the sweat glands were destroyed. Finally, there was loss of intra-epidermal nerve fibres in clinically unaffected leprosy skin. This finding may be
Fig. 5 Skin biopsies immunostained with antibodies to PGP, the pan-neuronal marker. Unaffected (A) and affected (B) sites from the same patient showing normal sweat gland morphology in both, but a complete loss of PGP-immunoreactive innervation in the affected site. An affected site (C) showing severe inflammation with gland destruction and only remnants of PGP-immunoreactive nerve (arrow). Immunoperoxidase with nuclear counterstain (Neutral Red). Scale bar = 100 μm.
helpful in detecting sub-clinical nerve involvement, and has implications for sensory biology in general. Loss of pain and temperature sensation, and sweating, have long been recognized as the earliest neuropathic symptoms in leprosy. Since then, it has been established, using immunohistochemistry, that cutaneous unmyelinated afferent and autonomic nerve fibres contain neuro-effector peptides, in addition to the classic neurotransmitters (see Anand, 1996, 1997). Investigation of skin biopsies by immunohistochemistry with antibodies to the pan-neuronal marker PGP 9.5 and neuropeptides in previous studies has shown that PGP-immunoreactive nerves may persist in lesion sites, although reduced compared with non-leprous control subjects, and that sensory and autonomic neuropeptide immunoreactivity was reduced or completely abolished in nearly all leprosy cases and animal models (Anand et al., 1983a; Karanth et al., 1989; Antunes et al., 1997). Our results of PGP 9.5-immunoreactive fibres in affected sites are in accord with previous studies. Apart from the correlations with quantitative testing, the present study extends the findings of previous studies by comparing affected and clinically unaffected sites from the same patient. We have shown that clinically uninvolved leprosy sites have a normal distribution of dermal PGP-immunoreactive nerves, correlating with normal sensory axon-reflex vasodilatation. As fibres positive for calcitonin gene-related peptide in sub-epidermal regions mediate axon-reflex vasodilatation, we have used these in our clinically unaffected site biopsies, and have found their abundance to be similar to non-leprosy control skin (our unpublished observations).

Intra-epidermal nerve fibres were lost in the majority of cases in the present study, in clinically unaffected as well as affected skin, and may provide a test for detecting sub-clinical nerve involvement in leprosy cases and their contacts. Studies of finger/toe vasomotor testing with laser Doppler fluxmetry may show sub-clinical deficits in leprosy (Wilder-Smith and Wilder-Smith, 1996; Abbot et al., 1996), but these studies lack a morphological correlation, and deficits are likely to result from more proximal nerve rather than cutaneous involvement. The role of intra-epidermal nerve fibres is at present unknown. Our findings support the proposal that while they are not necessary for sensation in normal conditions, they are recruited in inflammation. In animal models of skin inflammation, increased nerve growth factor production by keratinocytes plays a critical role in the development of hyperalgesia and the sprouting of sensory fibres, including intra-epidermal nerve fibres (see Anand, 1996, 1997). As leprosy skin, including clinically unaffected skin, appears to have reduced nerve-growth-factor immunostaining in epidermis (Facer et al., 1998), this may explain the loss of intra-epidermal fibres.

It has been proposed that a number of changes in leprosy skin may result from a deficit in nerve growth factor, and that local treatment with recombinant human nerve growth factor may improve nociception and sweating (Anand, 1996, 1997). The findings of the present study will help in the selection of patients with reduced but detectable nerve function, and no loss of target organ function, for such trials of treatment. The tests we describe can also help monitor selective functional effects of treatment, prior to histological studies which detect structural effects.

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