Immunohistochemical detection of PGL-1, LAM, 30 kD and 65 kD antigens in leprosy infected paraffin preserved skin and nerve sections

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Summary A panel of lipid, carbohydrate and protein antibodies were optimized for use in detecting *M. leprae* antigens in paraffin embedded material. Skin and nerve biopsies from 13 patients across the leprosy spectrum were studied. All antibodies detected antigen in tissues with a BI >1. Phenolic-glycolipid was not detected in bacteriologically negative tissue but lipoarabinomanan (LAM) and protein antigens were detected. Staining with LAM was strongest and gave least background. The transfer of this immunohistochemical technique to paraffin embedded material will allow examination of tissue with better morphology and from clinics without access to tissue freezing facilities.

Introduction

The paucity of *M. leprae* bacilli in tuberculoid leprosy disease spectrum in leprosy poses diagnostic difficulties histologically. One potential solution to this problem is to use antibodies against *M. leprae* antigens in immunohistochemical techniques to detect individual antigens rather than bacilli. *M. leprae* has protein, lipid and carbohydrate antigens. The lipid and carbohydrate antigens are found in the *M. leprae* cell wall. Phenolic glycolipid is unique to *M. leprae*, lipoarabinomannan is a soluble polysaccharide and is present in other mycobacteria. *M. leprae* has numerous protein antigens; the 65 kDa is a heat shock protein and 30 kDa is a secreted antigen. Immunohistochemical studies have been done on leprosy infected tissue using anti-BCG monoclonals[1,2] and monoclonal antibodies (mAbs) directed against phenolicglycolipid-1 (PGL-1),[3-5] lipoarabinomannan (LAM)[6,5] and different protein epitopes of *M. leprae*[3,6-10,14] to detect these antigens. Most of these studies were performed on skin biopsies; only three workers have reported results of staining nerve biopsies for

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leprosy antigens.[1,3,4] Few studies have been performed on paraffin embedded material in which tissue morphology is better preserved. Wang *et al.*[5] have demonstrated PGL-1 and LAM-B in paraffin preserved leprosy material, but *M. leprae* protein epitopes have not been demonstrated in paraffin preserved leprosy specimens. In this study, a panel of mAbs was used for an immunohistochemical analysis of paraffin embedded nerve and skin biopsies. Intensity and differential staining patterns were analysed and compared with bacillary load and macrophage numbers.

Materials and methods

MATERIAL

Tissue specimens from 13 leprosy patients were obtained from the Hospital for Tropical Diseases (London) and the Dhoolpet Leprosy Research Centre (Hyderabad, India). Patients had been classified clinically and histologically using the Ridley–Jopling classification. Bacillary indices were counted at the London School of Hygiene and Tropical Medicine using the Ridley logarithmic scale. The group comprised two TT, four BT, three BL and four LL cases. In five cases nerve biopsy material was also available (one BT, one BL and three LL cases). Control skin sections were taken from psoriasis (as a control for skin inflammation) and leishmaniasis (granulomatous skin control) cases (Departments of Dermatology and Pathology, Academical Medical Centre, University of Amsterdam, The Netherlands) and normal skin and nerve (post mortem) tissue (University College Hospitals, London, UK). All material was paraffin embedded and 5μm sections were cut.

IMMUNOHISTOCHEMICAL STAINING

Routine staining procedures (Dako) for immunohistochemistry of paraffin-embedded tissue sections were followed. Sections were incubated for 20 min in normal goat serum to block non-specific staining. The antibodies DZ-1 (PGL-1) and F26-7 (LAM) (Amsterdam) were diluted in a pre-evaluated dilution of 1:250 in PBS (phosphate buffered saline) and applied to the sections for 1 h, at room temperature. Pre-treatment in a trypsin-chymotrypsin bath for 15 min, at 37°C was required for sections stained with the antibodies 3A8 (30 kD) and F67-2 (65 kD). These antibodies were used at an optimal dilution of 1:75 and 1:1000 in PBS, respectively, with an overnight incubation at 4°C. The macrophage marker PG-M1 (Dako Cat

Table 1. Description of monoclonal antibodies used in this study: immunoglobulin class and specificity

mAbs	Ig class	Specificity	Source
DZ-1	IgG1	PGL-1	
F26-7	IgM	LAM	A. H. J. Kolk[12]
3A8	IgG1	30 kDa protein	P. K. Das[9,10,13]
F67.2	IgG1	65 kDa protein	A. H. J. Kolk[12,14]

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Table 2. Description of other mAbs tested and their *M. leprae* protein epitope specificity

mAb	Specificity	Dilutions tested in PBS	Ref
A2h11	30 kD	1:5, 1:10, 1:20, 1:40	10, 15, 16
Rb2	30 kD	1:5, 1:10, 1:20, 1:40	10, 15, 16
A3e2	30 kD	1:50, 1:100, 1:500, 1:1000	16
A4g4	30 kD	1:50, 1:100, 1:500, 1:1000	10, 16
Pe12	30 kD	1:50, 1:100	10, 15, 16
A3c12	26/27 kD	1:250, 1:500, 1:1000	10, 16
Ne5	65 kD	1:50, 1:100	17
Nd4	65 kD	1:50, 1:100	17

No M0876) specific for CD68 was diluted 1 in 50 and applied for 1 h at room temperature. The sections were subsequently incubated with biotinylated rabbit anti-mouse immunoglobulin (Dako Cat. No. E413) diluted 1:200 in PBS + 10% human AB serum for 30 min and finally incubated with streptABComplex/HRP (Dako No. K377) for 30 min. The peroxidase reaction was visualized using $3,3'$ diaminobenzidine tetrahydrochloride (DAB Sigmafast) for 5–10 min at room temperature and the sections were counterstained with haematoxylin. The specificity of the antibody staining was checked by omitting the primary antibody in at least one section from each patient.

Table 3. Staining intensity patterns of *M. leprae* monoclonal antibodies

Pat. no.	Classif.	Treatment status	BI	Antigen staining				Macrophages CD68
				PGL-1	LAM	30 kD	65 kD	
1.	TT	Untr	0	−	+	2+	+	2+
2.	TT act	Untr	0	−	+/−	+	2+	+
3.	BT	DDS/6 m	0	−	+/−	2+	3+	3+
4.	BT	DDS/1 m	0	−	+	+	2+	2+
5.	BT	Untr	0	+	+	2+	+	2+
6. A skin	BT	DDS/24 m	1+	+	+	+	2+	+
B nerve	BT	DDS/24 m	1+	−	+/−	−	+	+
7. A skin	BT/BL	MDT/irr	1+	+/−	+	+	+	+
B nerve	BT/BL	MDT/irr	2+	+	2+	−	+	2+
8.	BL	Untr	3+	+	+	+	+	nd
9.	BL act	DDS	3+	2+	2+	2+	+	2+
10. A skin	LL	DDS/12 m/MDT/23 m	2+	+	2+	2+	+	2+
B nerve	LL	DDS/12 m/MDT/23 m	3+	2+	2+	−	+/−	2+
11.	LL rel	unknown	3+	2+	3+	3+	+	4+
12. A skin	LL	DDS/irr	4+	2+	3+	3+	3+	3+
B nerve	LL	DDS/irr	5+	3+	4+	−	2+	4+
13. A skin	LL	DDS/2 m	5+	3+	3+	2+	2+	3+
B nerve	LL	DDS/2 m	3+	2+	2+	−	2+	2+
Leishmaniasis				−	−	−	−	nd
Psoriasis				−	−	−	+/−	nd
Normal skin				−	−	−	−	nd
Normal nerve				−	+/−	−	−	+/−

Patient 8 was in reversal reaction.
Grading of antigen staining: − negative, +/− weak, + fair, 2+ moderate, 3+ strong, 4+ very strong.
TT tuberculoid, BT borderline tuberculoid, BL borderline lepromatous and LL lepromatous leprosy.
Pat. no. patient number, *Class.* classification, *BI* bacillary index, *Untr* untreated, *DDS* dapsone, *m* month, *MDT* multidrug treatment, *irr* irregular, *nd* not done, *act* active, *rel* relapse.
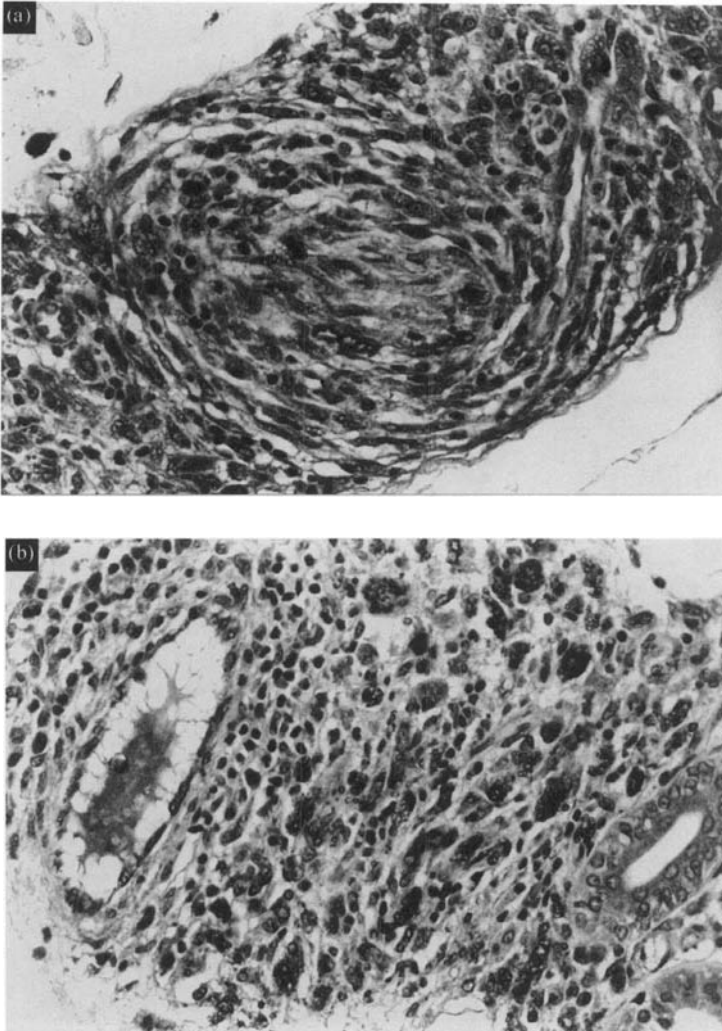
Figure 1. Staining patterns in LL patient (12) skin. All magnification ×400. (A) PGL staining, intracellular staining in macrophage. (B) LAM staining, strong vesicular staining in foamy macrophages. (C) 65 kDa (3A8) staining, cell surface staining is present. (D) Macrophage (CD68) marker.

Results

The characteristics of the four mAbs used in this study are listed in Table 1. At the start of the study, eight other mAbs (Table 2) were also tested on paraffin preserved material. However, no positive antigenic staining was found using dilutions around the optimal dilution for use on frozen material and following the above protocol.

Staining intensity patterns of the four *M. leprae* mAbs used in this study (directed against PGL-1, LAM, 30 kD and 65 kD) are shown in Table 3, together with data on bacillary index, CD68 positivity patterns and patient details. For grading purposes numbers of positively
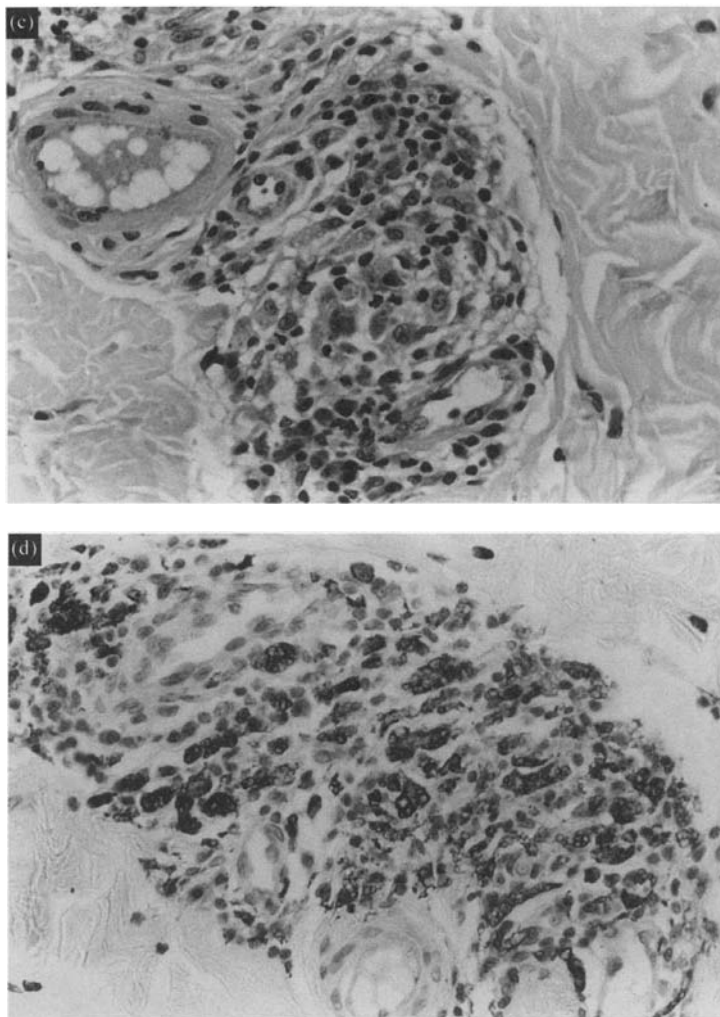
Figure 1. continued

stained infiltrating cells were counted semi-quantitatively by two observers. PGL-1 and LAM positive staining was found to be lowest at the tuberculoid pole and strongest at the lepromatous pole. Control samples were negative for antibody staining except for some 65 kD positivity in occasional subepidermal macrophages (identified by morphology) in psoriasis skin. In post mortem normal nerve tissue, a weak diffuse staining pattern was found within Schwann cells when staining for LAM.

SKIN

Both the PGL-1 and LAM antibodies gave intracellular granular staining in infiltrating macrophages and a clear vesicular staining pattern in foamy macrophages, although the latter
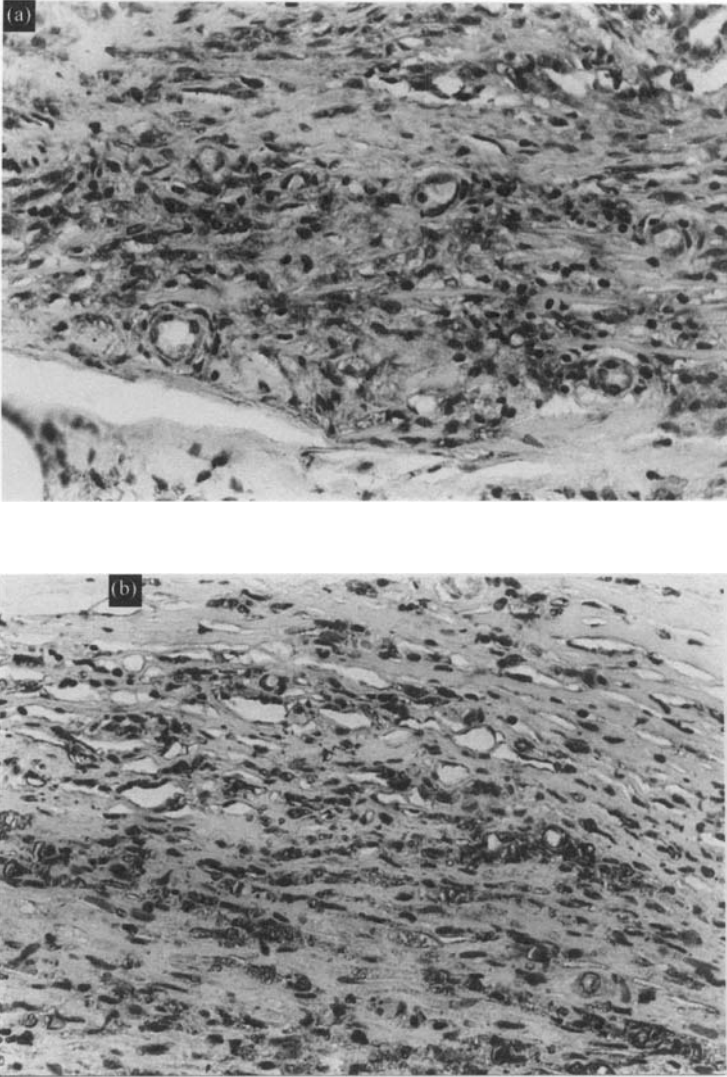
Figure 2. Staining patterns in LL (12) patient nerve. All magnification ×400. (A) PGL staining. (B) LAM staining in presumed Schwann cells. (C) 65 kDa (F67-2), weak cell surface staining. (D) Macrophage (CD68) infiltration in nerve.

pattern is mainly seen with LAM antibody staining (Figures 1A and B). With the PGL-1 antibody a weak diffuse staining was present in granulomata, this was not seen with LAM antibody staining. Occasionally some Langerhans cells stained positively with both the PGL-1 and LAM antibodies. With the 30 kD and 65 kD antibodies, staining of infiltrating cells was seen principally on cell membrane and nuclei with cytoplasm staining weakly (for 30 kD see Figure 1C). The intensity of the staining with the protein antibodies paralleled the macrophage infiltrate in the lesions. A diffuse granular staining observed within granulomas
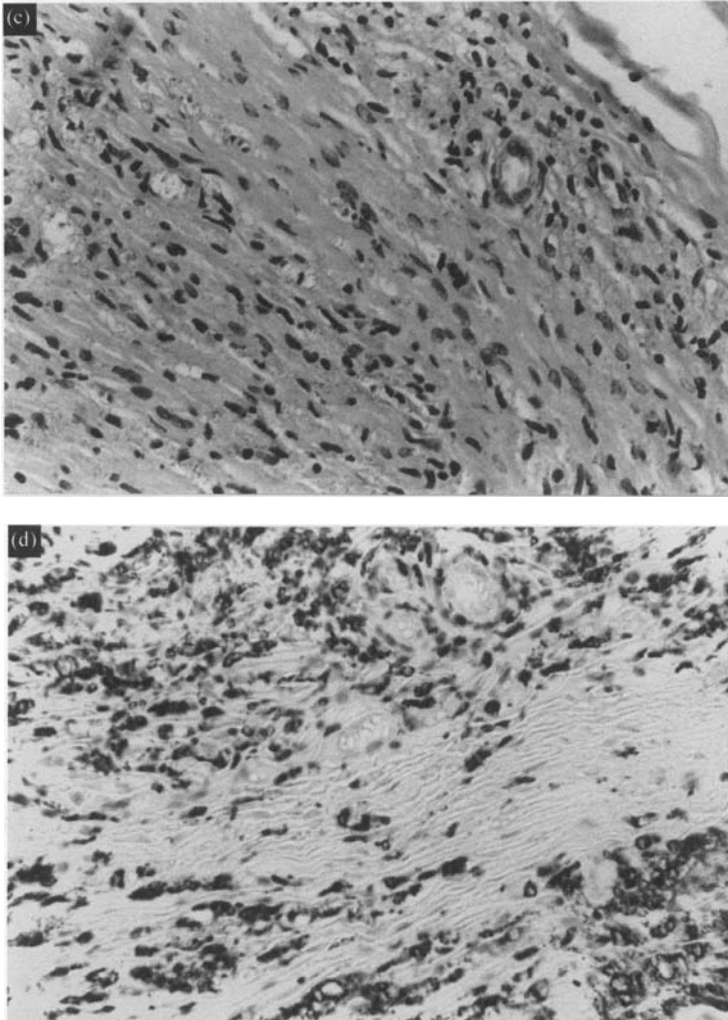
Figure 2. continued

was also seen with 30 kD, occasional background staining was present. The results of staining with 30 kD and 65 kD mAbs in skin suggested a parallel between the number of macrophages present and the intensity of staining.

NERVE

The 30 kD antigen could not be demonstrated in leprosy infected nerve tissue using the 3A8 antibody. For the other antigens the staining intensity paralleled the bacterial load and macrophage infiltrate (Figure 2D). In PGL-1 antibody stained samples PGL-1 was detected in infiltrating cells, mainly macrophages, in both granular and vesicular patterns (Figure 2A). The LAM antibody shows the same pattern, again staining foamy macrophages and with

considerably less background staining than the PGL-1 antibody (Figure 2B). The 65 kD mAb showed staining in infiltrating cells, but principally on the membranes and nuclei of those cells (Figure 2C). No identifiable bacteria were stained with the mAbs used in these skin and nerve sections. For both skin and nerve, positive staining was confined to areas where macrophages are found abundantly (Figures 1A–D).

In all infected skin tissue samples positive staining was also occasionally found in cells of the following cell lineages: melanocytes, vascular and sweat gland endothelial cells, hair follicles, and arrector pili muscle fibres. We have observed this epiphenomenon previously.

Discussion

In this study we have identified four mAbs that detect *M. leprae* antigens in paraffin embedded material. Using paraffin embedded material rather than frozen sections has the advantages that the morphology is much better preserved and specimens can be transported from remote areas for paraffin embedding.

The diagnosis of tuberculoid leprosy may be difficult when there are few granulomata, no definite nerve infiltration and no detectable acid fast bacilli. In such cases an antibody which stained antigen positive when Fite–Faraco staining was negative would be useful. In our study the LAM antibody would fulfil some of these criteria, because although both the PGL-1 and LAM antibody staining intensity correlated with the acid fast bacilli load the LAM antibody also stained positively in Fite–Faraco negative tissues whilst PGL-1 staining was negative at the tuberculoid pole. With the PGL-1 antibody (DZ-1) used here, four of the five TT/BT patients with a BI 0 had no detectable PGL-1, whether or not they had been treated. This contrasts with the work of Narayanan *et al.*[3] and Wang *et al.*,[5] who demonstrated the presence of PGL-1 antigens in bacteriologically negative tissue. The staining for LAM antigens was strongest and clearest, with less background than with the protein and lipid antibodies. One problem with the LAM antibody was that the normal nerve also stained positively with this antibody. This phenomenon has also been reported by Shetty *et al.*[4] and there may be cross reactive epitopes for LAM and nerve tissue. However, further work would be needed in a prospective study comprising patients with leprosy and other skin conditions to determine the sensitivity and specificity of this antibody.

In patients 6 and 10, who had both received many months of treatment (Table 3), staining for all antigens was still positive. This is in accordance with other workers who have found long term persistence of mycobacterial antigens in skin and nerve.[4,11]

The nerve sections used in this study could only be analysed for PGL-1, LAM and 65 kD staining, since the mAb against 30 kD (3A8) did not produce any positive staining in these sections. For PGL-1 and LAM, positive staining in nerve material when compared with staining in the corresponding skin correlated directly with the AFB load. Only PGL-1 showed an overall higher intensity staining in nerve material (three out of five cases), supporting the statement that mycobacterial antigen load is usually higher in nerve when compared with skin from the same patient.[4,11]

Using a panel of antibodies on paraffin embedded material permits future work assessing the amount and location of the different *M. leprae* carbohydrate, lipid and protein antigens in leprosy lesions. Defining *M. leprae* antigens in leprosy, in different disease types and at different sites may extend our understanding of the pathology of leprosy and may thus lead to new diagnostic and therapeutic modalities.

Acknowledgements

References

[1] Mshana RN, Humper DP, Harboe M, Belehu A. Demonstration of mycobacterial antigens in nerve biopsies from leprosy patients using peroxidase-antiperoxidase immunoenzyme technique. *Clin Immunol Immunopathol*, 1983; **29:** 359–368.

[2] Pagliari C, Duarte MIS, Sotto MN. Pattern of mycobacterial antigen detection in leprosy. *Rev Inst Med Trop Sao Paulo*, 1995; **37:** 7–12.

[3] Narayanan RB, Girdhar BK, Malaviya GN, Sengupta U. *In situ* demonstration of *Mycobacterium leprae* antigens in leprosy lesions using monoclonal antibodies. *Immunol Lett*, 1990; **24:** 179–183.

[4] Shetty VP, Uplekar MW, Anita NH. Immunohistological localization of mycobacterial antigens within the peripheral nerves of treated leprosy patients and their significance to nerve damage in leprosy. *Acta Neuropathol*, 1994; **88:** 300–306.

[5] Wang T, Izumi S, Butt KI *et al*. Demonstration of PGL-1 & LAM-B antigens in paraffin sections of leprosy skin lesions. *Jpn J Lepr (Nippon Rai Gakkai Zasshi)*, 1992; **61:** 165–174.

[6] Khanolkar SR, Mackenzie CD, Lucas SB *et al*. Identification of *Mycobacterium leprae* antigens in tissues of leprosy patients using monoclonal antibodies. *Int J Lepr*, 1989; **57:** 652–658.

[7] Naafs B, Kolk AHJ, Chin A Lien RAM *et al*. Anti-*Mycobacterium leprae* antibodies cross-react with human skin: an alternative explanation for the immune responses in leprosy. *J Invest Dermatol*, 1990; **94:** 685–689.

[8] Narayanan RB, Ramu G, Sinha S *et al*. Demonstration of *Mycobacterium leprae*-specific antigens in leprosy lesions using monoclonal antibodies. *Ind J Lepr*, 1985; **57:** 258–264.

[9] Rambukkana A, Das PK, Krieg S, Faber WR. Association of the mycobacterial 30-kDa region proteins with the cutaneous infiltrates of leprosy lesions. *Scand J Immunol*, 1992; **36:** 35–48.

[10] Rambukkana A, Burggraaf JD, Faber WR *et al*. The major mycobacterial secreted antigen 85 complex possesses epitopes that are differentially expressed in human leprosy lesions and *Mycobacterium leprae* infected armadillo tissues. *Infect Immun*, 1993; **61:** 1835–1845.

[11] Shetty VP, Suchitra K, Uplekar MW, Anita NH. Higher incidence of viable *Mycobacterium leprae* within the nerve as compared to skin among multibacillary leprosy patients released from multidrug therapy. *Lepr Rev*, 1997; **68:** 131–138.

[12] Anderson DC, Barry ME, Buchanan TM. Exact definition of species specific and crossreactive epitopes of the 65-kilodalton protein of *Mycobacterium leprae* using synthetic peptides. *J Immunol*, 1988; **141:** 607–613.

[13] Boddingius J, Dijkman H. Subcellular localization of *Mycobacterium leprae*-specific phenolic glycolipid (PGL-1) antigen in human leprosy lesions and in *M. leprae* isolated from armadillo liver. *J Gen Microbiol*, 1990; **136:** 2001–2012.

[14] Buchanan TM, Nomaguchi H, Anderson DC *et al*. Characterisation of antibody-reactive epitopes on the 65-kilodalton proteins of *Mycobacterium leprae*. *Infect Immunol*, 1987; **55:** 1000–1003.

[15] Rambukkana A, Das PK, Burggraaf JD *et al*. Identification and characterization if epitopes shared between the mycobacterial 65-Kilodalton heat shock protein and the actively secreted antigen 85 complex: their in situ expression on the cell wall surface of *Mycobacterium leprae*. *Infect Immun*, 1992; **60:** 4517–4527.

[16] Rambukkana A, Das PK, Burggraaf JD *et al*. Heterogeneity of monoclonal antibody-reactive epitopes on mycobacterial 30-kilodalton-region proteins and the secreted antigen 85 complex and demonstration of antigen 85B on the *Mycobacterium leprae* cell wall surface. *Infect Immun*, 1992; **60:** 5172–5181.

[17] Rambukkana A, Yong S, Das PK. Identification of a novel B-cell epitope of restricted specificity on the hsp 65-kDa protein of *Mycobacterium leprae*. Microbiol Immunol, 1991; **76:** 39–46.