Serological heterogeneity against various *Mycobacterium leprae* antigens and its use in serodiagnosis of leprosy patients

Leprosy is a chronic granulomatous disease caused by *Mycobacterium leprae* primarily affecting the peripheral nerves and skin. It still continues to be a health problem mainly in endemic countries such as India, Brazil, Myanmar, Madagascar, Nepal, Mozambique, Democratic Republic of Congo, Tanzania, Angola and the Central African Republic (World Health Organization, 2005). Worldwide, more than 400 000 new cases of leprosy are reported each year and most (more than 85.0 %) originate from the endemic countries mentioned above. Routinely, the disease is diagnosed clinically by specialists. However, due to integration of leprosy control programmes into general health services, the continued availability of sufficiently experienced health workers to diagnose leprosy is being threatened. Hence support tools are needed to ensure correct and timely diagnosis as well as treatment of leprosy patients even in the situation of non-availability of a specialist's diagnosis. Assays based on the detection of specific antibodies in serum are considered attractive due to their technical simplicity, speed, low cost and easy implementation under the conditions commonly encountered in developing countries. Today, the most extensively studied serological tests to detect *M. leprae* infection are IgM antibody detecting assays based on the *M. leprae*-specific phenolic glycolipid-I or its analogue (Oskam *et al.*, 2003). However, these tests are limited by the fact that about 60–85 % of the leprosy patients with low bacterial load (paucibacillary or PB patients) lack detectable serum antibody levels. We have therefore investigated the potential of other *M. leprae*-specific antigens, such as the *M. leprae* 45 kDa, ESAT-6 and CFP-10 antigens (Parkash *et al.*, 2006a, b, 2007). However, even though the 45 kDa antigen performed better than PGL-I in similar assays, our studies have indicated that these antigens in general are not recognized widely enough by serum

samples from leprosy patients, particularly PB patients (sensitivities ranged from 39 to 64 %), for the development of tests with sufficiently increased sensitivity to help diagnose all PB leprosy patients. In the present study, we have analysed the serological heterogeneity among assays based on PGL-I, 45 kDa antigen, ESAT-6 and CFP-10, and explored the performance of a combinatorial approach employing these four *M. leprae* antigens.

Approval for the study was obtained from our institute's (National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra) ethics committee for scientific research. After informed consent was obtained, blood samples from the patients and controls were collected. The diagnosis was based upon clinical criteria defined by the World Health Organization (1998) according to which persons with one or more characteristic symptoms, such as hypopigmented or reddened skin lesion(s) with definite loss of sensation, and/or involvement of the peripheral nerves as demonstrated by palpable thickening with loss of sensation, and skin smear positivity for acid-fast bacilli, were included as leprosy patients. The patients were classified (World Health Organization, 1988) as multibacillary (MB) or PB according to the presence or absence, respectively, of acid-fast bacilli (detected by Ziehl–Neelsen staining) in the skin smears. As controls, serum samples from clinically active pulmonary tuberculosis patients, patients with other skin diseases and healthy individuals were collected. In all, serum samples from 97 subjects were examined. The subjects included 17 smear-positive MB leprosy patients and 33 smear-negative PB leprosy patients. All of these patients were either untreated or had started therapy less than 1 month earlier. Serum samples from 13 clinically active pulmonary tuberculosis patients, 14 patients with other skin diseases and 20 healthy individuals were included as controls. After collecting,

serum samples were stored at -20°C until use.

Antibodies against PGL-I, the 45 kDa antigen, ESAT-6 and CFP-10 were detected by ELISAs as described elsewhere (Parkash *et al.*, 2006a, b, 2007). To score tests as positive, cut-off points for assays with various antigens were determined by constructing receiver operator characteristic curves, by calculating the per cent true-positives and false-positives at various absorbance values obtained by ELISAs. The absorbance value at which assay performance was optimal (true positives + true negatives/total sample number) was used as a cut-off point. A sample with an absorbance value of greater than the cut-off value was considered positive. The differences between groups with respect to the proportion of positively testing subjects were tested by the chi-square test.

The positivity patterns against various antigens among MB and PB patients are shown in Fig. 1(a) and Fig. 1(b), respectively. The positivity with all the antigens (PGL-I + 45 kDa + ESAT-6 + CFP-10) was higher (12/17) in the MB group and lower (1/33) in the PB group. When considering all leprosy patients (MB + PB), only 13 samples were positive by all the assays (PGL-I + 45 kDa + ESAT-6 + CFP-10). However, only 6, 2, 2 and 2 samples were exclusively positive by the assays based on the PGL-I, 45 kDa, ESAT-6 and CFP-10 antigens, respectively, whereas 2, 2, 0, 1, 7, 1, 0, 1, 2 and 0 samples were positive jointly by the assays based on the PGL-I + 45 kDa + ESAT-6, PGL-I + 45 kDa + CFP-10, PGL-I + ESAT-6 + CFP-10, 45 kDa + ESAT-6 + CFP-10, PGL-I + 45 kDa, PGL-I + ESAT-6, PGL-I + CFP-10, 45 kDa + ESAT-6, 45 kDa + CFP-10 and ESAT-6 + CFP-10 antigens, respectively. Thus the heterogeneity in MB patients was 29.4 % (5/17), which was lower than that (69.7 %; 23/33) among the PB group of patients. Concerning heterogeneity in

Fig. 1. Overlap and heterogeneity in antibody response against PGL-I, 45 kDa, ESAT-6 and CFP-10 antigens in sera of multibacillary (a) and paucibacillary (b) patients. Each column indicates one patient; each square indicates one test result with the corresponding antigen depicted on the extreme left side of the table. Shaded boxes, positive; white boxes, negative.

results with various antigens, it is possible that the genetic make-up of the host could contribute to the variation in the immune response. Moreover, the positivity with one antigen but lack of reactivity with another antigen could also be due to formation of immune complexes between respective antigens and antibodies thereby making the antigen-reactive sites non-available. Nevertheless, the occurrence of such possibilities with these antigens needs investigation.

The number and per cent positivity with all the assays are given in Table 1. When comparing the performances considering the two leprosy subgroups (MB and PB) separately, the positivity of the 45 kDa antigen for detection of MB patients was highest (17/17) among all the assays. Nonetheless, the sensitivity for detection of such patients did not differ statistically ($P > 0.05$) from the other assays, as the sensitivity was uniformly high (range 82.4–100.0 %) in those assays as well.

Concerning the performances of the assays with PB patients, again the highest sensitivity was with the 45 kDa antigen followed by PGL-I, ESAT-6 or CFP-10. The sensitivity of PGL-I was similar to that of the 45 kDa antigen, in line with our previous work (Parkash *et al.*, 2006a), whereas that of ESAT-6 or CFP-10 was statistically lower ($P < 0.01$) than that of the 45 kDa antigen but comparable ($P > 0.05$) with that of PGL-I. On considering all leprosy patients (i.e. MB + PB) together, positive proportions for antibodies against PGL-I, the 45 kDa antigen, ESAT-6 and CFP-10 were 27, 34, 20 and 20, respectively, compared to 2, 0, 0 and 1 out of the control group of 47 subjects. Thus the sensitivities and specificities of the various assays ranged from 40 to 68.0 % and 95.7 to 100.0 %, respectively. Among the various antigens studied here, the sensitivity of the 45 kDa antigen stood out as the best (68.0 %), being significantly higher ($P < 0.01$) than that of ESAT-6 (40.0 %) or CFP-10 (40.0 %) but comparable ($P > 0.05$) with that of PGL-I (54.0 %). However, the specificities of all assays were similar (range 97.9–100.0 %; $P > 0.05$).

Prompted by the heterogenic antibody response described above, we analysed the results in a combined fashion (i.e. positive by any of the assays) for detection of leprosy patients (Table 1). Considering MB and PB groups separately and combining the results of all the assays together (PGL-I + 45 kDa + ESAT-6 + CFP-10), the

Table 1. Results of ELISA for detection of anti-PGL-I, anti-45 kDa, anti-ESAT-6 and anti-CFP-10 antibodies

Group	No. of patients	PGL-I [no. positive (%)]	45 kDa [no. positive (%)]	ESAT-6 [no. positive (%)]	CFP-10 [no. positive (%)]	No. positive by any of the assays (%)
Leprosy						
Multibacillary	17	16 (94.1)	17 (100.0)	14 (82.4)	14 (82.4)	17 (100.0)
Paucibacillary	33	11 (33.3)	17* (51.5)	6 (18.2)	6 (18.2)	24† (72.7)
Total	50	27 (54.0)	34‡ (68.0)	20 (40.0)	20 (40.0)	41§ (82.0)
Controls						
Tuberculosis	13	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0)
Other skin diseases	14	1 (7.1)	0 (0.0)	0 (0.0)	0 (0.0)	1 (7.1)
Healthy	20	1 (5.0)	0 (0.0)	0 (0.0)	1 (5.0)	2 (10.0)
Total	47	2 (4.3)	0 (0.0)	0 (0.0)	1 (2.1)	3 (6.1)

*Significantly ($P < 0.01$) different when compared to ESAT-6 or CFP-10.

†Significantly ($P < 0.01$) different when compared to PGL-I, ESAT-6 or CFP-10.

‡Significantly ($P < 0.01$) different when compared to ESAT-6 or CFP-10.

§Significantly different when compared to PGL-I ($P < 0.01$), ESAT-6 ($P < 0.001$) or CFP-10 ($P < 0.001$).

sensitivity (17/17; 100.0 %) for detection of MB patients did not differ ($P > 0.05$) from that of the assays based on PGL-I (16/17; 94.1 %), ESAT-6 (14/17; 82.5 %) or CFP-10 (14/17; 82.5 %). However, for PB leprosy patients, the combined (PGL-I + 45 kDa + ESAT-6 + CFP-10) approach yielded a sensitivity of 72.7 % (24/33), resulting in an increase of 40.4, 21.2, 54.5 and 54.5 % over that of PGL-I ($P < 0.01$), the 45 kDa antigen ($P > 0.05$), ESAT-6 ($P < 0.01$) and CFP-10 ($P < 0.01$) as single antigens, respectively. When all leprosy patients (MB + PB) were taken together, the combined sensitivity with all the antigens was 82.0 %, implying a 28.0, 14.0, 42.0 and 42.0 % increase in sensitivity over that of the anti-PGL-I ($P < 0.01$), 45 kDa ($P > 0.05$), ESAT-6 ($P < 0.001$) and CFP-10 ($P < 0.001$) antibody-detecting assays, respectively. Although the increase in the sensitivity of 45 kDa antigen was comparable ($P > 0.05$) to that of PGL-I, it was significantly different from that of ESAT-6 ($P < 0.05$) or CFP-10 ($P < 0.05$). Interestingly, the specificity invariably remained more than about 94.0 % in all the assays.

Thus the combinatorial approach, considering various antigen-based assays, appears to be the most sensitive one. Adopting this approach could lead to detection of 21.2–54.5 % more of the smear-negative PB leprosy patients who otherwise would have been missed with any of the above-mentioned single-antigen-based assays. Hence, we consider this combined approach to be promising for application for serodiagnosis of leprosy.

Nevertheless, for better diagnostic applicability, the combined sensitivity needs to be increased further, while maintaining overall specificity. In this context, the availability of the complete gene sequence of *M. leprae* (Cole *et al.*, 2001) may help to identify additional *M. leprae* antigens with serodiagnostic potential. Recently, several groups have reported a number of new *M. leprae*-specific antigens and peptides capable of stimulating B and T cells (Geluk *et al.*, 2005; Spencer *et al.*, 2005; Araoz *et al.*, 2006; Reece *et al.*, 2006; Groathouse

a new immunological tool(s) for diagnosis of leprosy patients. The results of the present study provide a new outlook for the development of improved methods by exploring new antigens in combinatorial multiplex studies to improve the serodiagnosis of leprosy.

Acknowledgements

We are grateful to the Netherlands Leprosy Relief Foundation, and the Q.M. Gastmann Wichers Foundation, which sponsored part of this research. We are also grateful to Dr P. J. Brennan and Dr J. S. Spencer from Colorado State University, USA, and NIH/NIAID Contract No. 01 AI 25469 for providing the ND-O-HSA and to LEPRO, UK, for financial support. Thanks to Ms Astha Nigam, Department of Biochemistry, School of Life Sciences, Dr B. R. Ambedkar University, Agra, India, for collecting the samples and to Dr B. K. Girdhar, National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra, India, and Dr M. L. Arora, S.N. Medical College, Agra, India, for providing samples and other relevant details of the patients. Thanks to Mr Amit Kumar for his help. O.P. thanks his wife, Mrs Vimla Devi, for her patience and co-operation in his efforts.

Om Parkash,¹ Ajay Kumar,² Richa Pandey,¹ Bhaweshwar K. Girdhar,¹ Kees L. M. C. Franken³ and Tom H. M. Ottenhoff³

¹National JALMA Institute for Leprosy and Other Mycobacterial Diseases, TajGanj, Agra-1, India

²Department of Biomedical Sciences, Bundelkhand University, Jhansi (U.P.), India

³Department of Immunohematology and Blood Transfusion, and Department of Infectious Diseases, Leiden University Medical Center, The Netherlands

Correspondence: Om Parkash (om1234@gmail.com)

Araoz, R., Honore, N., Banu, S., Demangel, C., Cissoko, Y., Arama, C., Uddin, M. K. M., Abdul, S. K. H., Monot, M. & other authors (2006). Towards an immunodiagnostic test for leprosy. *Microbes Infect* 8, 2270–2276.

Cole, S. T., Eiglmeier, K., Parkhill, J., James, K. D., Thomson, N. R., Wheeler, P. R., Honoré, N.,

Garnier, T., Churcher, C. & other authors (2001). Massive gene decay in the leprosy bacillus. *Nature* 409, 1007–1011.

Geluk, A., Klein, M. R., Franken, K. L., van Meijgaarden, K. E., Wieles, B., Pereira, K. C., Bühner-Sékula, S., Klatser, P. R., Brennan, P. J. & other authors (2005). Postgenomic approach to identify novel antigens of *Mycobacterium leprae* for improved immunodiagnosis of *M. leprae* infection. *Infect Immun* 73, 5636–5644.

Groathouse, N. A., Amin, A., Marques, M. A., Spencer, J. S., Gelber, R., Knudson, D. L., Belisle, J. T., Brennan, P. J. & Slayden, R. A. (2006). Use of protein microarrays to define the humoral immune response in leprosy patients and identification of disease-state-specific antigenic profiles. *Infect Immun* 74, 6458–6466.

Oskam, L., Slim, E. & Buhner-Sekula, S. (2003). Serology in leprosy research and control: recent developments, strengths, limitations and prospects: a state of the art overview. *Lepr Rev* 74, 196–205.

Parkash, O., Kumar, A., Astha, N., Franken, K. L. M. C. & Ottenhoff, T. H. M. (2006a). Evaluation of recombinant serine-rich 45kDa antigen (ML0411) for detection of antibodies in leprosy patients. *Scand J Immunol* 64, 450–455.

Parkash, O., Kumar, A., Nigam, A. & Girdhar, B. K. (2006b). Detection of antibodies against *Mycobacterium leprae* culture filtrate protein-10 in leprosy patients. *J Med Microbiol* 55, 1337–1341.

Parkash, O., Pandey, R., Kumar, A. & Kumar, A. (2007). Performance of recombinant ESAT-6 antigen (ML0049) for detection of leprosy patients. *Lett Appl Microbiol* 44, 524–530.

Reece, S. T., Ireton, G., Mohamath, R., Guderian, J., Goto, W., Gelber, R., Groathouse, N., Spencer, J., Brennan, P. & Reed, S. G. (2006). ML0405 and ML2331 are antigens of *Mycobacterium leprae* with potential for diagnosis of leprosy. *Clin Vaccine Immunol* 13, 333–340.

Spencer, J. S., Dockrell, H. M., Kim, H. J., Marques, M. A., Williams, D. L., Martins, M. V., Martins, M. L., Lima, M. C., Sarno, E. N. & other authors (2005). Identification of specific proteins and peptides in *Mycobacterium leprae* suitable for the selective diagnosis of leprosy. *J Immunol* 175, 7930–7938.

World Health Organization (1988). WHO Expert Committee on Leprosy, Geneva. *World Health Organ Tech Rep Ser* 768, 1–51.

World Health Organization (1998). WHO Expert Committee on Leprosy, Geneva. *World Health Organ Tech Rep Ser* 874, 1–43.

World Health Organization (2005). Global strategy for further reducing the leprosy burden and sustaining leprosy control activities. *Lepr Rev* 76, 265–287.