Detection of antibodies against *Mycobacterium leprae* culture filtrate protein-10 in leprosy patients

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The prevalence of IgG antibodies against *Mycobacterium leprae* recombinant culture filtrate protein-10 (rCFP-10) was investigated in serum samples from 56 leprosy patients, 15 tuberculosis (TB) patients, 14 other skin-diseased patients and 20 healthy subjects. On classifying the patients into bacterial index (BI)-positive and BI-negative groups, the assay showed 83.3% (15/18) sensitivity for detection of BI-positive leprosy patients. On the other hand, the sensitivity for detection of BI-negative patients was 18.4% (7/38). None of the 15 TB patients and 14 other skin-diseased patients was positive; however, only one out of 20 healthy individuals was positive, indicating that antibody response to culture filtrate protein-10 (CFP-10) was highly specific (98.0%; 48/49). Statistically, the performance of the CFP-10-based assay was found to be comparable ($P > 0.05$) with that of an anti-phenolic glycolipid-I (PGL-I) antibody-detecting assay. Thus, *M. leprae* CFP-10 is potentially a specific antigen for measuring antibody response in BI-positive leprosy patients. Being a secreted antigen, CFP-10 may act as a marker for the viability of *M. leprae* inside the host, and hence its serological potential is worth exploring for application in monitoring the response of patients with BI-positive leprosy (a highly infectious form) during the course of chemotherapy. When comparing the bacteriological and serological results, an agreement of 82.1% showed that seropositivity to *M. leprae* CFP-10 corresponded well with bacteriological criteria. Hence, CFP-10 seems to be a suitable antigen for classification of leprosy patients into BI-positive and BI-negative groups.

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INTRODUCTION

Leprosy, caused by the bacterium *Mycobacterium leprae*, is an infectious and complex mycobacterial disease whose manifestations and complications are determined by the immune response. The number of recorded leprosy cases worldwide has fallen dramatically, from approximately 10 million cases in 1985 to about 0.46 million at the beginning of 2004. The majority (84%) of these cases were limited to nine developing countries, of which India had the greatest number (World Health Organization, 2005). The big fall in leprosy cases is mainly attributed to the use of multidrug therapy (MDT) in treating patients. However, to date, there is no clear evidence of an impact of the introduction of MDT on the rate of occurrence of new cases, as more than 0.5 million new cases are detected every year. This implies that leprosy transmission is somehow continuing, and new cases will probably continue to occur, at least in endemic

countries, for several more years to come. Therefore, a strategy to identify leprosy cases in the early stages and to treat them could contribute greatly to the interruption of leprosy transmission and thereby to the control of leprosy.

Over the past several years, one of the major areas of leprosy research has remained the identification of *M. leprae* antigens and their immunological characterization, with the aim of exploring their potential to act as reagents for serodiagnosis of leprosy patients (Hunter & Brennan, 1981; Hunter *et al.*, 1990; Young *et al.*, 1992; Thole *et al.*, 1995; Triccas *et al.*, 1998; Reece *et al.*, 2006). The recent publication of the genome sequences of *M. leprae* (Cole *et al.*, 2001) and *Mycobacterium tuberculosis* (Cole *et al.*, 1998) have provided ample opportunity to identify new proteins of *M. leprae*. During this process, the homologue of *M. tuberculosis* culture filtrate protein-10 (CFP-10; Rv3874) (Berthet *et al.*, 1998), a 10 kDa protein, has been identified in *M. leprae* (ML0050). The extent of identity between *M. leprae* CFP-10 and its counterpart in *M. tuberculosis* is only 36%. Geluk *et al.* (2004) have shown the *M. leprae* CFP-10 protein to be a potent antigen that stimulates T-cell-dependent gamma interferon production in a large

Abbreviations: BI, bacterial index; CFP-10, culture filtrate protein-10; MDT, multidrug therapy; PGL-I, phenolic glycolipid-I; rCFP-10, recombinant CFP-10; ROC curve, receiver operator characteristic curve; TB, tuberculosis.

proportion of individuals exposed to *M. leprae*. However, 60 % of 12 leprosy patients responded to the *M. tuberculosis* CFP-10, and 100 % of nine tuberculosis (TB) patients responded to *M. leprae* CFP-10, including TB patients who had never been to leprosy-endemic areas. This indicates fairly high cross-reactivity at the T-cell level between *M. leprae* and *M. tuberculosis* CFP-10. On the other hand, Spencer *et al.* (2004), using a set of sera of eight TB and four lepromatous leprosy patients, have shown that there is little or no cross-reactivity at the antibody level with heterologous peptides or the whole CFP-10 protein. In this study, we have assessed the suitability of the recombinant CFP-10 (rCFP-10) of *M. leprae* for serology in leprosy patients.

METHODS

Subjects. Serum samples from 56 leprosy patients were collected from patients attending the outpatient department (OPD) of the National JALMA Institute for Leprosy and other Mycobacterial Diseases, TajGanj, Agra, India. The diagnosis was made by clinical criteria (World Health Organization, 1998). Persons with one or more of the following characteristic symptoms were included as leprosy patients: 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. All of these patients were either untreated or had started therapy less than 1 month earlier. The bacterial index (BI) in leprosy patients was determined by observing acid-fast bacilli in the skin smears, before starting treatment by MDT. All smear-positive cases were grouped as BI positive and those that were smear negative were taken as BI negative. On the basis of the BI results, leprosy patients were divided into two groups: BI positive (18) and BI negative (38). Serum samples from 15 clinically active pulmonary TB patients attending S. N. Medical College, Agra, India, 14 other skin-diseased patients attending the OPD of the National JALMA Institute for Leprosy and other Mycobacterial Diseases, and 20 healthy individuals were included as controls. The approval for the study was obtained from the ethics committee for scientific researches of the institute, and informed consent was obtained from all subjects enrolled in the study.

ELISA for detection of antibodies. Antibodies against rCFP-10 were detected by ELISA, employing purified *M. leprae* rCFP-10 antigen that was produced using *Escherichia coli* as an expression host. The antigen was purified by nickel affinity chromatography from the supernatant of the sonicated *E. coli* cells (Spencer *et al.*, 2004). Flat-bottomed 96-well ELISA plates (Maxisorp, Nunc) were coated with 50 µl per well of the rCFP-10 antigen at a concentration of 5 µg ml⁻¹ in normal saline, and incubated overnight at 4 °C. Alternate wells were coated with coating solution (normal saline) without antigen, and this served as a control for background signal. The solutions in the wells were discarded, and the wells were then blocked with 1 % BSA in Tris-buffered saline (TBS, pH 8.0), followed by incubation for 1 h at 37 °C. After three washes with Tween-20 (0.1 %)-supplemented TBS (TBS-T), serum samples (diluted 1:25 in TBS-T) were added to the wells. One serologically positive and one serologically negative sample were run as test controls. Subsequently, the plate was incubated for 2 h at 37 °C; this was followed by three further washes with TBS-T. Next, peroxidase-labelled rabbit polyclonal anti-human IgG (Dakocytomation) diluted 1:6000 in TBS-T was added (50 µl per well) to the wells, and the plate was incubated for an additional 1 h at 37 °C. The washing stage was repeated as before, and 50 µl per well of tetramethyl benzidine substrate (Genei) was added. After 20 min colour development,

the enzymic reaction was stopped by adding 50 µl per well of 10 % H₂SO₄. Finally, the A₄₅₀ values of the wells were read in a microplate reader (Spectra Max, Molecular Devices). The specific A₄₅₀ values were calculated by subtracting the values measured in wells coated with coating solution (non-specific binding) from those in the wells coated with antigen. The cut-off points were calculated using receiver operator characteristic (ROC) curve analysis (Fig. 1). Samples with A₄₅₀ values above the cut-off point (0.150) were considered positive.

The samples were tested (singly) three times in three independent experiments. The mean of the results of three independent experiments was considered for data analysis. The test result of a sample was included in the study only in cases where the findings of the assay (in terms of scoring the results as positive or negative) were similar in at least two independent tests.

RESULTS AND DISCUSSION

The single greatest need in leprosy research remains the development of diagnostic tool(s) that can easily detect early infection, and thereby allow intervention before the onset of the disease. Serology is thought to be an affordable tool for this purpose. Consequently, a number of protein antigens of *M. leprae* have been identified, of which the 35 kDa protein and phenolic glycolipid-I (PGL-I) have been studied most widely (Parkash, 2002; Oskam *et al.*, 2003). In the present study, we suggest that *M. leprae* CFP-10 is a worthwhile antigen for use in serological studies of leprosy patients.

The intensities of the antibody response to CFP-10 antigen in serum samples belonging to various groups are shown in

Fig. 1. ROC curve of ELISA for measurement of antibodies against rCFP-10 antigen in sera of leprosy patients. This was done by calculating the percentage of true positives for leprosy and false positives for controls at various cut-off points expressed in terms of A₄₅₀ values. The optimal cut-off point (0.150) is indicated by an arrow.

Table 1. Mean A_{450} values obtained with ELISA for detection of anti-CFP-10 antibodies in leprosy patients and controls

Subjects	No. of patients	Mean $A_{450} \pm SD$	Range
Leprosy patients			
BI positive	18	0.551 ± 0.340	0.029–1.012
BI negative	38	0.096 ± 0.216	–0.283–0.707
Controls			
TB patients	15	-0.007 ± 0.074	–0.164–0.121
Other skin-diseased patients	14	-0.044 ± 0.067	–0.144–0.077
Healthy subjects	20	0.037 ± 0.062	–0.175–0.146

Table 1. The antigen was most strongly recognized by BI-positive patient sera, with a mean $\pm$ SD A_{450} of 0.551 ± 0.340 . Mean levels were significantly higher ($P < 0.001$) in BI-positive patients than in BI-negative patients, healthy individuals, other skin-diseased patients and TB patients. The extent of CFP-10 antigen recognition by BI-negative patients was also significantly ($P < 0.05$) higher than that of TB patients and other skin-diseased patients. Although the mean A_{450} was slightly higher in the BI-negative group than that of the healthy control group, the difference was statistically non-significant ($P > 0.05$).

To score the test result as positive, a cut-off point was defined by ROC curve analysis from the antibody response against CFP-10 (Fig. 1), by calculating the percentages of true-positive and false-positive values at various A_{450} values. The A_{450} value at which assay performance was optimal (true positives + true negatives/total sample number) was used as a cut-off point (0.150).

Using the above-defined cut-off point, the majority (83.3 %) of the BI-positive patients gave positive reactions to CFP-10 (Table 2), whereas 18.4 % of the BI-negative patients were positive. Taking all the leprosy patients together, the positivity was found to be 39.3 %. However, three BI-positive (1 +, 1 +, 5 +, respectively) patients were negative by CFP-10-based assay. Furthermore, none of the TB patients and other skin-diseased patients was positive, whereas only one of the 20 healthy individuals was positive

by anti-CFP-10 assay, indicating a high (97.96 %; 48/49) specificity for the assay. With the anti-PGL-I antibody ELISA, the positivity rates in the BI-positive and BI-negative groups were determined to be 94.4 % (17/18) and 36.8 % (14/38), respectively, with a specificity of 95.9 % (47/49). Taking all leprosy patients together, the sensitivity of the anti-PGL-I antibody assay was found to be 55.4 % (31/56) (detailed data not shown). Thus, the sensitivity attained by the anti-CFP-10 assay was lower than that of anti-PGL-I antibody ELISA; however, statistically, the performances of both assays were comparable ($P > 0.05$). Furthermore, in the case of the anti-PGL-1 antibody assay, only one BI-positive (3 +) patient was negative. Fifteen samples negative for anti-CFP-10 antibody were found to be positive by anti-PGL-I antibody assay, and six samples negative by anti-PGL-I antibody assay were found to be positive by the anti-CFP-10 antibody assay. This indicates the existence of heterogeneity in the recognition of the antigens by some individuals, which could be due to their different genetic make up.

We next analysed the results from both the assays in a joint fashion. When all leprosy patients (BI positive and BI negative) and both the assays were considered together, the overall sensitivity increased to 66.1 % (37/56), implying a 10.7 % increase in sensitivity over the anti-PGL-I antibody assay. Considering BI-positive and BI-negative cases separately, the sensitivities of the anti-PGL-I antibody assay increased to 100 % (18/18) and 50 % (19/38), giving

Table 2. ELISA results in leprosy patients classified using BI as the criterion

Subjects	No. of serum samples	No. positive	No. negative	Sensitivity (%)
Leprosy patients				
BI positive	18	15	3	83.3
BI negative	38	7	31	18.4
Total	56	22	34	39.3
Controls				
TB patients	15	0	15	0
Other skin-diseased patients	14	0	14	0
Healthy subjects	20	1	19	5.0
Total	49	1	48	2.04

increases of 5.6 and 13.2%, respectively. However, specificity remained high (93.9%; 46/49). Thus, in all the cases, this combinatorial approach increased, though in a statistically non-significant manner ($P > 0.05$), the sensitivity of the anti-PGL-I antibody assay, with a marginal decrease (2.04%; $P > 0.05$) in specificity.

The present data substantiates the earlier findings of Spencer *et al.* (2004) in that the serological responses of TB and leprosy patients were quite specific, showing none of the cross-reactivity found against the heterologous proteins at the B-cell level. The lack of reactivity with samples from TB patients indicates that despite 40% identity between *M. leprae* CFP-10 and *M. tuberculosis* CFP-10, there is an absence of cross-reactivity at the B-cell-response level with homologous CFP-10 in *M. tuberculosis*. This is unlike the situation for *M. leprae* CFP-10 cross-reactivity with *M. tuberculosis* at the T-cell level (Geluk *et al.*, 2004). The reason for this discrepancy in the results could be due to the different ways in which B and T cells recognize antigens. B cells recognize antigens in solution or on cell surfaces, but always in their native conformations, whereas the T-cell receptor (TCR) recognizes antigen presented in association with major histocompatibility complex (MHC) molecules on cell surfaces. Antigens recognized by T cells have often been degraded or processed in some way, so that the determinant recognized by the TCR is only a small fragment or peptide of the original antigen.

In leprosy patients, the lower positivity (in comparison to the PGL-I-based assay) against CFP-10 is probably due to a weak antibody response in some individuals due to their genetic make up and/or the weak antigenicity of the CFP-10 antigen. Alternatively, the lower serological positivity could be the result of complex formation of the anti-CFP-10 antibody with the corresponding antigen, thus making the antibody unavailable in the assay. This speculation is supported by earlier reports (Ramanathan *et al.*, 1984; Patil *et al.*, 1986) that demonstrate the existence of mycobacterial antigens in immune complexes in serum samples from leprosy patients. Nevertheless, the occurrence of these possibilities needs investigation.

Early detection of failure of treatment in leprosy patients could open the possibility of putting patients on alternative effective treatment. During treatment of patients with multibacillary leprosy, the anti-PGL-I antibody levels generally decrease in parallel with the decrease in bacterial load (Klatser *et al.*, 1989; Douglas *et al.*, 1989). In some patients, anti-PGL-I titres decline rapidly in a linear fashion, while in others, the antibody levels drop gradually and persist even after the patient is supposedly cured of disease (Gelber *et al.*, 1989). Some investigators suggest that this may be due to dead or non-multiplying dormant bacteria that are still present (World Health Organization, 1988; Gelber *et al.*, 1989; Meeker *et al.*, 1990) in the body. Since CFP-10 is a secretory protein (Spencer *et al.*, 2004), it would only be produced by viable and growing *M. leprae* inside the host. Therefore, the detection of anti-CFP-10 antibodies

might act as a better or alternative prognostic marker for monitoring the response to treatment in leprosy patients. Hence, it would be interesting to evaluate CFP-10, either alone or as a complementary reagent with other antigen(s), for monitoring chemotherapy in multibacillary leprosy patients.

Classification of leprosy patients into BI positive and BI negative determines the duration of their treatment. Misclassification leads to increased risk of relapse due to insufficient treatment if a BI-positive patient is classified as BI negative. This also prolongs the time that the patient is infectious. When the leprosy patients were grouped on the basis of the smear-positivity criterion into BI-positive and BI-negative groups, the agreement (82.1%) between the bacteriological classification and serological results was substantial (kappa value of 0.61). This indicates that CFP-10 could prove to be a suitable antigen for sero-classification of leprosy patients into BI-positive and BI-negative types.

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