Detection of *Mycobacterium leprae* Antigens in the Sera of Leprosy Patients by Sandwich Immunoradiometric Assay Using Monoclonal Antibodies

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An immunological technique for demonstration of *Mycobacterium leprae* antigens in sera was developed by using specific as well as cross-reactive monoclonal antibodies. The sandwich immunoradiometric assay which we developed is a simple, robust assay that is sensitive to the nanogram level. Sera from 72 leprosy patients were screened for the presence of antigen by this assay. A total of 69% of untreated tuberculoid leprosy patients showed 35-kDa antigen positivity, and 45% of these patients showed anti-35-kDa antibody positivity. Consistently higher antigen positivity rates for the 35-, 12-, and 30- to 40-kDa components of *M. leprae* were observed in lepromatous leprosy patients than in tuberculoid leprosy patients. During the course of therapy the antigen positivity rate gradually declined, and the antigen could not be detected in any of the 15 patients with subsided cases of leprosy. As antigen is presumably in excess before the antibody response is evoked, our experimental approach for antigen detection is likely to be useful by itself or along with antibody detection for diagnosis of early leprosy.

Human hosts respond to exposure to *Mycobacterium leprae*, the causative agent of leprosy, by developing an antibody response and cell-mediated immunity. The humoral response does not have a significant role in combating the infection, but it can be utilized for diagnosing the infection.

The antibody response to *M. leprae* infection is directed toward several determinants, the majority of which are shared with other species of mycobacteria; only a few of these determinants are specific to *M. leprae* (1). The currently used serodiagnostic assays include a fluorescent leprosy antibody absorption assay, a radioimmunoassay, a phenolic glycolipid-based assay, a monoclonal antibody (MAb)-based immunoradiometric assay, and an MAb-based enzyme-linked immunosorbent assay. These assays are based on detection of antibodies which are considered to be specific against surface components, lipids, and proteins of *M. leprae* (1). However, these assays do not show good correlation with early forms of the disease.

The main deficiency of the immunodiagnostic tests listed above is that they detect only the free antibodies against a defined antigen and the antibody molecules which are complexed with antigen escape detection. Therefore, in an antigen-antibody equivalence situation and in situations in which antigen is in excess, the antibody assays fail to indicate positivity and give false-negative results. In view of this deficiency, assays in which antigen and antibody are detected may prove to be more sensitive diagnostic tests.

Antigen-based diagnostic assays are already used for various diseases, such as hepatitis (2), tuberculous meningitis (3), and filariasis (10). A recent study of acquired immune deficiency syndrome (14) has indicated that in this infection the antigen in serum is detectable in 100% of the cases before patients become antibody positive. Such systematic estimates for circulating *M. leprae* products and the implications of these estimates for diagnosis of leprosy have not been

determined previously. Although *M. leprae*-specific lipid antigen has been detected in sera of leprosy patients, mainly in patients with severe forms of the disease (4, 15), such assays are not available for detection of antigen in the early stages of the disease due to the low level of sensitivity of the technique. In this study, complexed antigens in sera were first released by sodium dodecyl sulfate (SDS) treatment (11), and a set of MAbs (MAbs ML04, ML06, and ML34) were used to detect the presence of the corresponding *M. leprae* antigens (35-, 12-, and 30- to 40-kDa, respectively) by a double-antibody sandwich immunoradiometric assay. The sera were also subjected to an antibody detection test by using the same MAbs.

MATERIALS AND METHODS

Blood samples for this study were collected from 72 leprosy patients with active disease and 15 patients with subsided disease who attended the inpatient and outpatient departments of the Central JALMA Institute for Leprosy, Agra, India, and from 36 controls. The control subjects included 22 healthy laboratory volunteers and 14 patients with active pulmonary tuberculosis from the Tuberculosis Demonstration Centre, Agra. The sera were separated from blood samples and stored at −70°C. The leprosy patients were clinically classified according to the Ridley-Jopling classification (12). Of the 72 patients with active leprosy, 36 belonged to the tuberculoid group (group TT/BT); 16 of these patients were untreated, and 20 had had treatment for about 2 months to 1 year at the time of sample collection. The remaining 36 patients with active leprosy belonged to the lepromatous group (group BL/LL); 18 of these patients were untreated, and 18 were undergoing treatment. Eight of the 15 patients with subsided cases belonged to group TT/BT, and 7 belonged to group BL/LL.

Serum samples were subjected to SDS treatment to release the complexed antigens (11). To 1 ml of test serum 250 μl of 10% SDS was added, and the preparation was mixed thoroughly before incubation for 2 h at 37°C. The SDS was

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later removed by dialyzing the samples for 48 h against normal saline to avoid interference by the SDS in the antigen detection assay (11). The samples were then lyophilized, reconstituted to their original serum volumes, and tested for antigen.

MAbs. MAbs ML04, ML06, and ML34, which bind to epitopes on 35-kDa (protein), 12-kDa (protein), and 30- to 40-kDa (polysaccharide) soluble components of *M. leprae*, respectively (9), were labeled with [125]I (BARC, Bombay, India) by the Iodogen method (6).

***M. leprae* antigen.** The cell extract (soluble antigen) of armadillo-derived purified *M. leprae* (5) was obtained from the Immunology of Leprosy (World Health Organization) Bank, London, England, and was used as the antigen.

Double-antibody sandwich immunoradiometric assay. The double-antibody sandwich immunoradiometric assay is a solid-phase antigen capture assay. An antibody molecule which is bound to a solid phase is used to capture the antigen from the test sample, and another antibody molecule, which is radiolabeled, is used to probe the captured antigens. The principle used in this assay is that the antigen should have at least two sites to combine with two antibody molecules.

Wells of flexible microtiter plates (type U96; Dynatech) were coated with MAbs (50 μl per well) by overnight incubation at 4°C in a moist chamber. The free sites of the wells were blocked with 100 μl of 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (pH 7.4) for 2 h at room temperature. Test samples (50 μl) were applied to the wells in duplicate, and the preparations were incubated for 2 h at 37°C in a moist chamber. Each plate was then washed with PBS, and 50 μl of the corresponding ^{125}I-labeled MAb was added to each well. The plate was incubated again for 2 h and washed three times with PBS, and counts for each well were determined with a model NE-1600UK gamma counter.

Samples which exhibited counts that were more than the mean count plus 2 standard deviations for the normal samples were considered positive for antigen.

Antigen standard curve. Standard samples with known concentrations of antigens were made by incorporating *M. leprae* antigen into pooled serum samples from normal healthy volunteers from areas where mycobacterial diseases are not endemic. These samples were subjected to the sandwich immunoradiometric assay by using each MAb, and standard curves were constructed.

Immunoradiometric antibody detection assay. The immunoradiometric antibody detection assay was performed by the method of Hewitt et al. (7), with slight modifications. For this antibody competition test, flexible 96-well microtiter plates were coated with armadillo-derived *M. leprae* antigen (5) (50 μl per well; 50 μg/ml) by overnight incubation at 4°C. Unbound antigen was later removed by washing with PBS. The wells were blocked with 100 μl of 2% BSA per well in PBS for 2 h at room temperature. Unbound BSA was removed by dumping each plate and then patting it on tissue paper. A 25-μl portion of each serum sample diluted to log₅ in BSA was added to wells in duplicate, and the preparations were incubated for 1 h at 37°C in a moist chamber; this was followed by addition of 25 μl of ^{125}I-labeled MAb ML04, ML06, or ML34 to the wells. Further incubation was carried out for 2 h at 37°C. The whole plate was then washed three times with PBS and blotted dry, and radioactive counts were determined with a gamma counter. Samples from normal individuals showed 30 to 40% inhibition of binding of ^{125}I-labeled MAbs at a 1:5 dilution of serum; hence, samples showing more than 50% inhibition were considered antibody positive (7). The percent inhibition was calculated on the
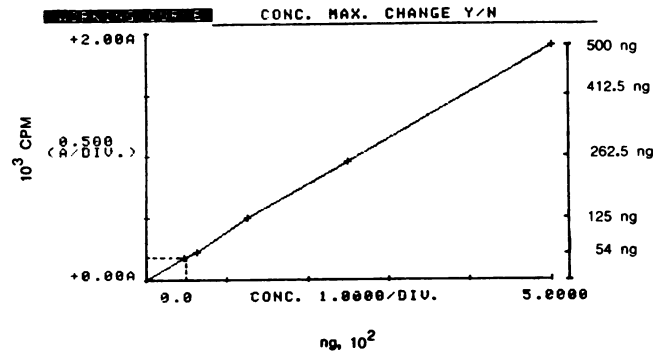

FIG. 1. Standard curve for the 35-kDa protein antigen assay. The curve was constructed by incorporating *M. leprae* antigen into serum. The sensitivity limit of the assay was 54 ng, as calculated on the basis of counts per minute.

basis of counts per minute (cpm), as follows: % inhibition = 100 − % binding = 100 − cpm in test well/100% binding × 100, where 100% binding was defined as the level of binding of ^{125}I-labeled MAb directly to an antigen-coated well after blocking with 2% BSA in PBS.

RESULTS

Standard curves constructed by using MAbs ML04 and ML34 worked in a dose-dependent manner (Fig. 1 and 2). However, a standard curve could not be constructed by using MAb ML06. This may have been due to the presence of a single epitope on the 12-kDa molecule.

35-kDa antigen capture. An *M. leprae* quasispecific epitope present on the 35-kDa cytoplasmic protein was captured by MAb ML04 (Fig. 3).

Eleven of 16 (69%) untreated and 4 of 20 (20%) treated subjects in the tuberculoid group were positive for the 35-kDa antigen. In the lepromatous group 13 of 18 (72%) untreated and 8 of 18 (44%) treated patients were antigen positive. The cutoff point for this antigen was about 200 cpm as determined by the sandwich immunoradiometric assay. None of the 14 tuberculosis patients and 22 healthy individuals was positive for the 35-kDa antigen. The highest level of antigen (4.8 μg/ml) was recorded in an untreated patient belonging to group BT. The level of sensitivity of this 35-kDa antigen assay was 54 ng, as calculated by using the standard curve.
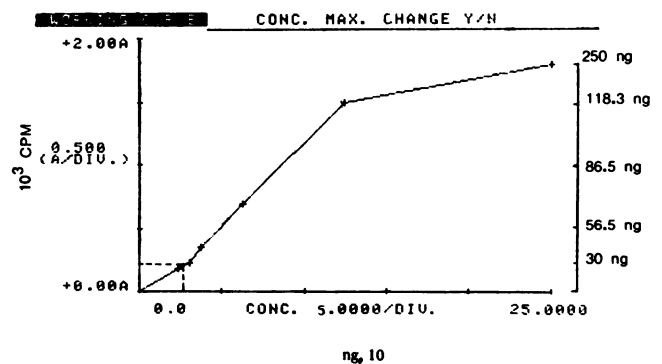

FIG. 2. Standard curve for the 30- to 40-kDa polysaccharide antigen assay. The curve was constructed by incorporating *M. leprae* antigen into serum. The sensitivity limit of the assay was 30 ng, as calculated on the basis of counts per minute.
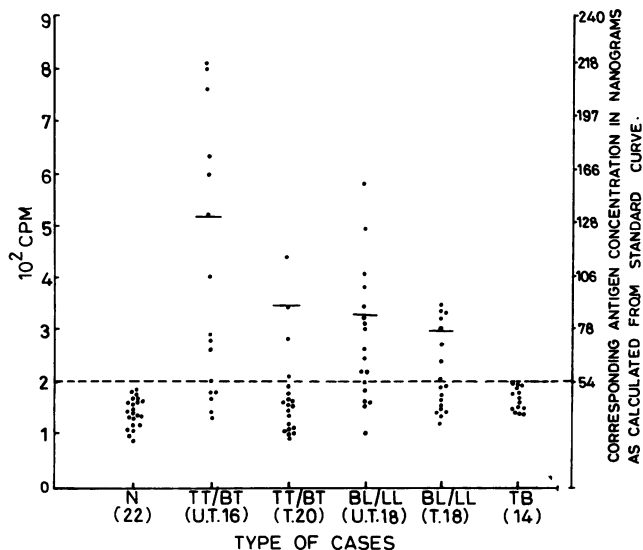
FIG. 3. 35-kDa *M. leprae* protein antigen in SDS-treated serum samples. The dashed line indicates the cutoff point for antigen-positive subjects, and the solid lines indicate the mean values for antigen-positive subjects. The P values for the differences between values for healthy control sera [N (22)] and values for disease sera, as evaluated by using the unpaired t test, are as follows: tuberculoid untreated [TT/BT (U.T.16)], $P < 0.001$; tuberculoid under treatment [TT/BT (T.20)], $P > 0.05$; lepromatous untreated [BL/LL (U.T.18)], $P < 0.005$; lepromatous under treatment [BL/LL (T.18)], $P > 0.01$; and tuberculosis [TB (14)], $P > 0.05$.

12-kDa antigen capture. An *M. leprae*-specific epitope present on the 12-kDa cytoplasmic protein was captured by MAb ML06 (Fig. 4).

Seven of 16 untreated (44%) and 11 of 20 treated (55%) subjects in the tuberculoid group of leprosy patients were positive for the 12-kDa antigen. In the lepromatous group 14 of 18 (78%) patients in both the treated and untreated groups were positive for this antigen. A total of 22 normal subjects tested by this assay were all below the cutoff point (190 cpm) and hence were negative for the 12-kDa antigen. However, 2 of the 14 pulmonary tuberculosis patients were positive for the 12-kDa antigen as determined by this assay. The sensitivity of the assay could not be determined because of a lack of dose-dependent behavior of this antigen molecule in the standard curve. The highest value for the 12-kDa antigen (550 cpm) was recorded in an untreated patient belonging to group LL.

30- to 40-kDa antigen capture. An epitope that was highly cross-reactive with other mycobacteria and was present on the 30- to 40-kDa polysaccharide component of the cell wall was captured by MAb ML34 (Fig. 5).

Three of 16 (19%) untreated and 3 of 20 (15%) treated subjects in the tuberculoid group were positive for this polysaccharide antigen. In the lepromatous group 13 of 18 (72%) untreated and 2 of 18 (11%) treated patients were positive for this component; 4 of the 14 (29%) tuberculosis patients were also found to be positive for this cross-reactive mycobacterial component by the assay. None of the 22 healthy individuals showed antigen positivity as they were all below the cutoff point (210 cpm). The highest level of antigen (1.2 μg/ml) was recorded in an untreated patient belonging to group LL. The sensitivity level of this polysaccharide antigen assay was 30 ng as calculated by the standard curve.
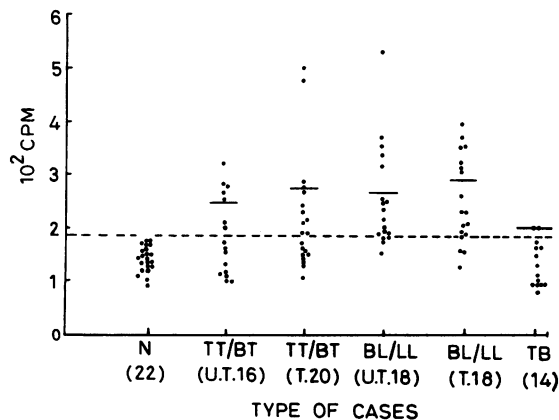

FIG. 4. 12-kDa *M. leprae* protein antigen in SDS-treated serum samples. The dashed line indicates the cutoff point for antigen-positive subjects, and the solid lines indicate the mean values for antigen-positive subjects. The P values for the differences between values for healthy control sera [N (22)] and values for disease sera, as evaluated by using the unpaired t test, are as follows: tuberculoid untreated [TT/BT (U.T.16)], $P < 0.05$; tuberculoid under treatment [TT/BT (T.20)], $P < 0.01$; lepromatous untreated [BL/LL (U.T.18)], $P < 0.001$; lepromatous under treatment [BL/LL (T.18)], $P < 0.001$; and tuberculosis [TB (14)], $P > 0.05$.

Antigen capture assays performed with samples from patients with subsided cases of leprosy (eight group TT/BT and seven group BL/LL cases) did not reveal positivity for any of the antigens (12, 35, and 30 to 40 kDa) (Fig. 6).

Anti-35-kDa antibody. Seven of 16 subjects (44%) in the untreated tuberculoid group, 18 of 18 subjects (100%) in the untreated lepromatous group, and 9 of 15 subjects (60%) in the group with subsided cases of leprosy exhibited antibodies against the 35-kDa component of *M. leprae*. None of the
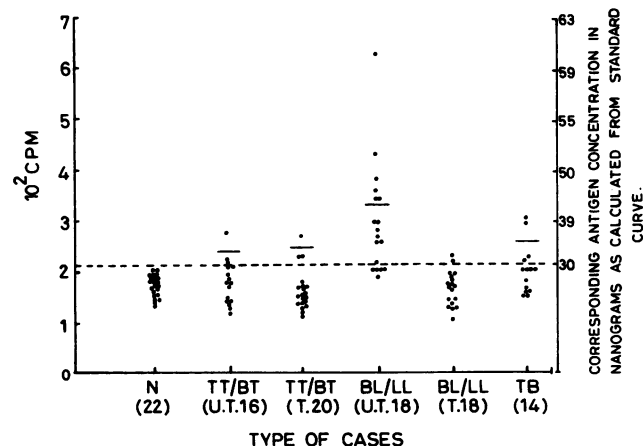

FIG. 5. 30- to 40-kDa polysaccharide antigen in SDS-treated serum samples. The dashed line indicates the cutoff point for antigen-positive subjects, and the solid lines indicate the mean values for antigen-positive subjects. The P values for the differences between values for healthy control sera [N (22)] and values for disease sera, as evaluated by using the unpaired t test, are as follows: tuberculoid untreated [TT/BT (U.T.16)], $P > 0.05$; tuberculoid under treatment [TT/BT (T.20)], $P > 0.05$; lepromatous untreated [BL/LL (U.T.18)], $P < 0.001$; lepromatous under treatment [BL/LL (T.18)], $P > 0.05$; tuberculosis [TB (14)], $P > 0.05$.
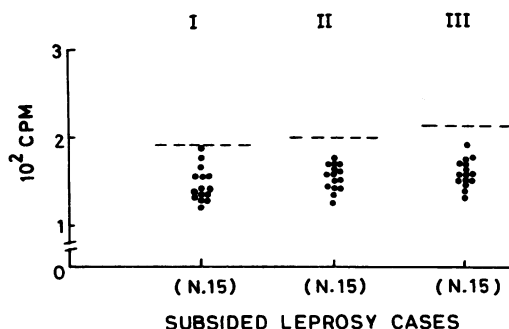
I   II   III

FIG. 6. 12-kDa (I), 35-kDa (II), and 30- to 40-kDa (III) *M. leprae* antigen assays. The dashed lines indicate the cutoff points for the various antigen detection assays. The values for sera from patients with subsided cases of leprosy were all insignificant compared with the values for healthy control sera.

14 tuberculosis patients and 22 healthy individuals exhibited antibodies against this 35-kDa component (Table 1).

Anti-12-kDa antibody. Two of 16 subjects (13%) in the untreated tuberculoid group, 10 of 18 subjects (56%) in the untreated lepromatous group, and 5 of 15 subjects (33%) in the group with subsided cases of leprosy exhibited antibodies against the 12-kDa component of *M. leprae*. However, none of the 14 tuberculoid patients and 22 healthy individuals exhibited antibodies against this 12-kDa component (Table 1).

Anti-30-to-40-kDa antibody. Three of 16 subjects (19%) in the untreated tuberculoid group, 16 of 18 subjects (89%) in the untreated lepromatous group, and 6 of 15 subjects (40%) in the group with subsided cases of leprosy exhibited antibodies against the 30- to 40-kDa component of *M. leprae*; 13 of 14 tuberculosis patients (93%) also exhibited antibodies against this antigen. However, none of the 22 healthy individuals was positive for antibodies against this component (Table 1).

DISCUSSION

The antigen capture sandwich immunoradiometric assay could conveniently be used to detect protein and polysaccharide antigens in the sera of leprosy patients. This assay works in a dose-dependent manner by using MAbs ML04 and ML34. The MAb ML06-based assay did not work in a similar fashion because of the presence of a single antigenic epitope on the 12-kDa molecule, and hence a standard curve for the 12-kDa antigen could not be constructed. However, antigen from the sera of leprosy patients could work in the assay, thereby suggesting that there are differences between in vivo (human) processed antigen and armadillo-derived *M.*

leprae sonicated antigen with regard to distribution of antigenic epitopes.

A straight line was obtained as a standard curve when we used MAb ML04, which belongs to the immunoglobulin G class, whereas the curve was not linear with MAb ML34, which belongs to the immunoglobulin M class. This could be because the pentamer structure of immunoglobulin M reacted with antigenic epitopes of closer proximity, giving rise to increased steric hindrance.

The phenolic glycolipid antigen assay, a chemical assay in which chloroform-methanol extraction is used, is less sensitive in early forms of the disease. Our assay is relatively simple and can demonstrate antigen in a high proportion of patients at the tuberculoid pole of the disease. The level of antibody response to the 35-kDa epitope was about 45% for the tuberculoid type of the leprosy, whereas the 35-kDa antigen was detected at a level of about 70% in the same group of patients. This indicates that there is weak antibody response at the tuberculoid form of the disease. Some of the serum samples from this group showed the presence of direct antigen, without SDS treatment, thus giving evidence of antigen excess in these subjects. However, the level of antigen positivity was not higher than the level of antibody positivity in the lepromatous group. The level of antigen positivity determined by the sandwich immunoradiometric assay was higher than the level determined by gel diffusion after similar treatment of serum samples for antigen release (11).

Although the antigen levels obtained were quite variable from group to group and antigen to antigen, higher antigen positivity rates were uniformly observed in the lepromatous group than in the tuberculoid group for all three antigens which were detected. Similarly, higher antibody positivity rates were observed in the lepromatous group than in the tuberculoid group for all three antibodies. We also observed that the levels of antigen positivity declined in most of the cases during the course of antileprosy therapy. However, not much difference was observed in antibody levels after treatment (data not shown). The decline in the antigen level for the 30- to 40-kDa polysaccharide component was much more pronounced after therapy was begun.

The 12-kDa protein antigen, which is (grossly) about 10% of the total *M. leprae* protein (8), seems to be a weak immunogen as most of the subjects in the tuberculoid group were found to be negative for antibodies against this component, whereas only 60% of the lepromatous untreated subjects showed antibody positivity (Table 1). However, we observed that the 12-kDa protein could be detected in lesser quantities than the 35-kDa protein (5% of the total *M. leprae* protein) in early forms of infection. Of the three antibody responses studied, antibodies against the 35-kDa antigen

TABLE 1. Comparison of antigen positivity results in sera with and without SDS treatment and with antibody positivity

Type of cases	Level of antigen positivity before SDS treatment			Level of antigen positivity after SDS treatment			Level of antibody positivity in neat serum		
	35 kDa	12 kDa	30 to 40 kDa	35 kDa	12 kDa	30 to 40 kDa	MAb ML04	MAb ML06	MAb ML34
TT/BT	3/16 (19)[a]	0/16 (0)	3/16 (19)	11/16 (69)	7/16 (44)	3/16 (19)	7/16 (44)	2/16 (13)	3/16 (19)
BL/LL	1/18 (6)	1/18 (6)	5/18 (28)	13/18 (72)	14/18 (78)	13/18 (72)	18/18 (100)	10/18 (56)	16/18 (89)
Subsided leprosy	0/15 (0)	0/15 (0)	0/15 (0)	0/15 (0)	0/15 (0)	0/15 (0)	9/15 (60)	5/15 (33)	6/15 (40)
Pulmonary tuberculosis	0/14 (0)	0/14 (0)	3/14 (21)	0/14 (0)	2/14 (0)	4/14 (29)	0/14 (0)	0/14 (0)	13/14 (93)
Healthy volunteers	0/22 (0)	0/22 (0)	0/22 (0)	0/22 (0)	0/22 (0)	0/22 (0)	0/22 (0)	0/22 (0)	0/22 (0)

[a] Number of samples positive/number of samples tested. The numbers in parentheses are percentages.

were observed in the majority of the individuals. This antigen has been reported to be highly immunogenic (9). The 30- to 40-kDa polysaccharide antigen seems to be a weak immunogen, especially in the early (tuberculoid) form of the disease.

Our assay detected antigens directly (without SDS treatment) in some paucibacillary cases which were found to be negative for the presence of antibody by the MAb-based competition assay. The currently available serological tests fail to reveal antibody positivity when there is excess antigen or until the antigen evokes the antibody response. However, in multibacillary cases antigen is detected in fewer individuals than antibody. Although the antigen detection assay has a higher capability for diagnosing paucibacillary cases on the basis of a higher level of positivity for antigen than for antibody, it may be very useful as an adjunct to the antibody detection assay for better serological diagnosis of leprosy.

In this study, although the patients were not followed until the subsidence of the disease, we observed that none of the 15 patients with subsided cases was positive for any of the antigens tested (35, 12, and 30 to 40 kDa). However, antibodies have been reported to occur in multibacillary cases even 5 to 10 years after the disease has subsided (13). The conventional skin smear negativity test may not be adequate to declare subsidence of infection as the skin smear test may not always give an indication of infection in deeper tissues. Therefore, detection of antigens in body fluids along with the skin smear test may serve as a better guide for declaration of subsidence of infection.

Detection of antibodies is a useful tool for clinical support of diagnosis, while detection of pathogen products confirms the presence of living pathogens. Therefore, detection of *M. leprae* antigens by our assay may provide an adjunct monitoring system for use with the antibody detection assay. Moreover, most of the serum samples from the tuberculoid group which were antigen positive were found to be negative for the presence of antibodies and vice versa. Such a distribution will be of further help in predicting infection in a larger number of subjects by using combined antigen and antibody detection tests.

ACKNOWLEDGMENTS

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