Serological Survey of Leprosy and Control Subjects by a Monoclonal Antibody-based Immunoassay[1]

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Leprosy continues to remain a serious public health problem in the developing countries and about 1.4 billion people are exposed to the risk of contracting the disease ([29]). The incidence of the disease could be reduced effectively by early diagnosis of subjects incubating the infection, who could then be put under close surveillance or given prophylactic treatment ([24]). Various workers have developed serological assays for leprosy. The major technical approaches have been: a) the fluorescent leprosy antibody absorption test (FLA-ABS) for the detection of *Mycobacterium leprae*-specific antibodies in sera preabsorbed with other species of mycobacteria for the elimination of crossreactive antibodies ([1, 2]); b) radioimmunoassay (RIA) of cross-absorbed sera for antibodies against the cell wall antigen 7 of *M. leprae* ([18]); and c) enzyme-linked immunoassay (ELISA) for antibodies against the phenolic glycolipid derived from cell walls of *M. leprae* ([4, 9, 30]).

Recently, we have reported the production of species-specific monoclonal antibodies against *M. leprae* ([21]) and subsequently developed, using the ML04 antibody, a serum competition RIA for the serology of leprosy ([26]). The reported preliminary data indicated that the competition test achieved the desired degrees of specificity and sensitivity. In this paper, we further report on serological data obtained from an extended collection of sera from groups of patients with various forms of leprosy and from nonleprosy control subjects.

MATERIALS AND METHODS

Sera. The test group consisted of 96 sera from untreated leprosy patients registered for treatment at the Central JALMA Institute for Leprosy (CJIL), Agra, India. Patients were classified by the Ridley-Jopling scale ([23]) and, accordingly, split into groups of LL/BL (58 cases), BB (8 cases), and TT/BT (30 cases) leprosy.

Sera from 43 apparently healthy household family members of proven leprosy cases (source cases) were also studied. These contacts were related (husband, wife, or children) either to multibacillary LL/BL/BB (28 sera) or to paucibacillary TT/BT (15 sera) source cases.

Sera representing the control groups were obtained from: a) healthy subjects, of whom 18 were staff members of CJIL and four were U.K. citizens on a visit to India; b) proven cases of active pulmonary tuberculosis attending the clinics of the TB Demonstration and Training Center, Agra; c) patients suffering from autoimmune disorders (sera showing antithyroid or antinuclear antibodies) attending the clinics of Safdarjang Hospital, New Delhi; and d) patients suffering from either breast or cervical cancer whose sera were provided by the Institute of Pathology, Safdarjang Hospital, New Delhi.

All control subjects were clinically examined and failed to manifest any signs of leprosy.

Antigen. *M. leprae* were separated from heavily infected armadillo livers by partition in an aqueous two-phase polymer system ([12]). The soluble antigen was prepared by sonication of bacilli at 20 kHz, 80 W using a Dawe Soniprobe type 7532A, immersed in iced water for 20 min, followed by centrifugation at 105,000 × *g* for 30 min, and filtration through a 0.22 μm membrane filter. The preparation (batch V6) at 1 mg

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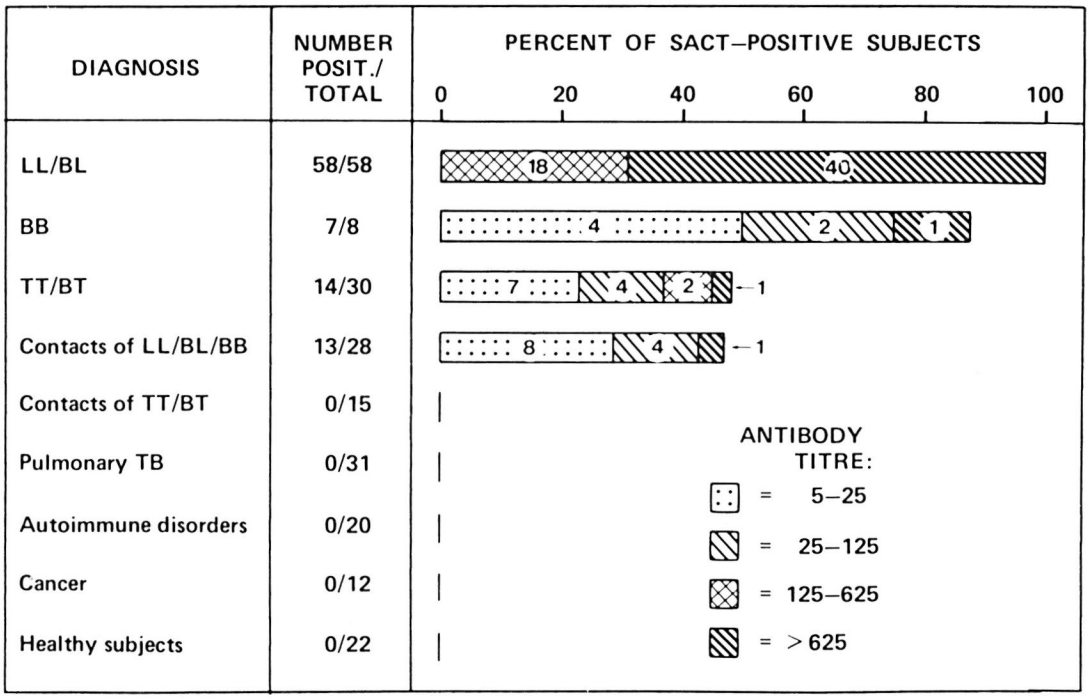

FIG. 1. The incidence and titer of *M. leprae*-specific (MY2a) antibodies in groups of leprosy patients and nonleprosy controls. Sera from a total of 224 subjects were tested in duplicate at four $\log_5$ serial dilutions by the SACT assay. Sera which failed to give 50% inhibition of ^{125}I-ML04 binding at the initial 1:5 serum dilution were scored as negative. Numbers of patients giving the corresponding ID_{50} antibody titers are encircled.

per ml concentration was kindly supplied by Dr. R. J. W. Rees, National Institute for Medical Research, London, U.K.

Monoclonal antibody. *M. leprae*-specific ML04 antibody ([21]) was produced as ascitic fluid in BALB/c mice. Immunoglobulins were precipitated from ascitic fluid by 18% sodium sulfate, dialyzed against phosphate buffered saline (PBS), pH 7.4, and stored lyophilized in vials. Batches of 50 μg of this antibody were labeled with Na ^{125}I (obtained from Isotope group, BARC, Bombay, India) using the "Iodogen" technique ([15]). After purification on a Sephadex G-50 column, more than 90% of the radioactivity was precipitated by trichloroacetic acid.

Serum antibody competition test (SACT). A modification of the already described technique ([20, 26]) was used. Polyvinyl chloride (PVC) microtiter plates (Dynatech, Inc.) were coated with 50 μl per well of the diluted soluble *M. leprae* antigen (50 μg/ml in PBS) overnight at 4°C. The plates were washed once with PBS; subsequently the wells were filled with a solution containing 2% bovine serum albumin (BSA), and 0.1% sodium azide in PBS (BSA-PBS) and stored covered with cellotape at 4°C until used.

Before the assay, the blocking solution was removed from the wells followed by two washings with PBS, and drying by patting on a tissue paper pad. $\log_5$ serum dilutions in BSA-PBS (four dilutions of each serum sample) were put into duplicate wells (25 μl/well) and incubated at 37°C in a humidified container for 1 hr. Afterward, ^{125}I-ML04 was added into each well (40,000 cpm in 25 μl BSA-PBS) and plates were further incubated for 2 hr at 37°C. After washing 5 times with PBS and drying the plates, individual wells were cut and radioactivities counted in a gamma counter (model NE 1600, Nuclear Enterprises, U.K.).

Expression of results. Mean binding of ^{125}I-ML04 to wells coated with BSA alone was taken as 0% and subtracted from each of the test binding values. Relative binding values for each serum dilution were calculated whereby binding of ^{125}I-ML04 in BSA-PBS alone to *M. leprae*-antigen-coated wells

was taken as 100% (1000 to 3000 cpm). The results were expressed in terms of ID_{50} values which represent the serum dilution causing 50% inhibition of ^{125}I-ML04 binding to the antigen-coated wells.

RESULTS

The prevalence and level of serum antibodies as determined by the SACT assay are expressed in Figure 1. All 58 LL/BL sera tested contained high titers of antibodies. However, the antibody response was considerably weaker in nonlepromatous forms. In the relatively small group (8 cases) of BB patients, antibody levels varied widely among the individuals. In the TT/BT form of the disease (30 cases), only 14 patients (47%) had demonstrable antibodies and of these, in 7 (23%) there were only low (ID_{50}-5–25) antibody levels.

Sera from clinically healthy family contacts of patients contained low antibody titers in 13 (46%) subjects, who were all related to multibacillary (LL/BL/BB) source cases. However, none of the 15 tested family contacts of TT/BT cases had demonstrable antibodies. As many as 85 sera were tested from nonleprosy control subjects, 81 of whom were residents of India. Although 31 of these were patients with pulmonary tuberculosis and 20 were positive for autoantibodies, all of the subjects were negative in the ML04-based SACT assay.

The SACT assay has been standardized for the titration of human sera, using four $\log_5$ dilutions. Representative curves demonstrating the various degrees of ^{125}I-ML04 binding inhibition by test sera from patients are demonstrated in Figure 2. Some control sera showed a low degree (<50%) of apparently nonspecific inhibition of ^{125}I-ML04 binding at 1:5 or lower serum dilutions. This was observed more frequently with sera from endemic areas, particularly from patients with tuberculosis. The effect can probably be attributed to steric hindrance caused by antibodies binding to sites in close proximity to the MY2a determinant.

DISCUSSION

The *M. leprae* bacillus is composed of several antigens (i.e., protein, glycolipid, and polysaccharide) which in turn are built from multiple distinct antigenic determinants (epitopes) ([3, 5, 10, 17, 19]). It seems conceivable

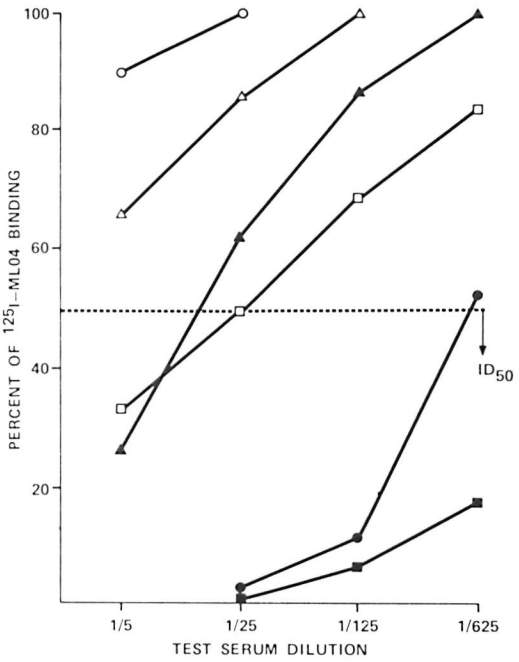

FIG. 2. Demonstration of representative titration curves of ^{125}I-ML04 inhibitory serum antibodies. Maximally inhibitory nonendemic (○) or endemic (△) control sera; antibody positive contact of a LL patient (□); antibody positive sera from TT/BT (▲), BL (●) or LL (■) patients.

that the antibody response which ensues as a result of infection is directed toward several determinants, of which the majority is shared with other species of mycobacteria and only the minority is specific for *M. leprae.* Using various antigenic preparations, several authors have clearly demonstrated a striking increase in antibody level in patients at the lepromatous pole and a lower, marginal, or no increase at the tuberculoid pole of the disease ([1, 4, 18, 30]). Thus, the enhanced antibody response in LL/BL patients is well demonstrable even by semiquantitative assays based on estimations with a single dilution rather than serial dilutions of tested sera. However, problems may arise in a) the definition of the margin which defines the border between the "background" and the "increased" antibody level; and b) the elimination of "false positive" values which may result from excessive stimulation with environmental mycobacteria or with *M. tuberculosis.*

The SACT assay resolves both problems listed above on the grounds of its specificity,

which is restricted to a single antigenic determinant (MY2a) of *M. leprae*. This determinant is apparently immunodominant in LL/BL patients, since sera from all 58 tested patients contained very high antibody levels. Moreover, the reliable specificity of the SACT assay was demonstrated here by the absence of "false positive" values in any of the tested sera from various control groups comprised of patients with active tuberculosis, autoimmune disorders, and cancer. It has been discussed previously that inhibition of monoclonal antibody binding by sera from patients with schistosomiasis could be attributed to either antibodies or to circulating antigen ([22]). The possible contribution of *M. leprae* antigen in either free or in immune complex ([6]) form to the SACT results will need to be addressed in future experiments.

The results of this study showed a contrast between the high titers (>625 in 69%) of LL/BL and the lack of response (53.3%) or low titer (23.3%) of antibody in the TT/BT group. The latter result may be attributed to the restriction of the assay to anti-MY2a-specific antibodies. However, it would seem unlikely that TT/BT patients produce antibodies of different specificity, since other authors have found only low or absent antibody responses in most patients at the tuberculoid pole of the disease spectrum ([27, 28]). The failure to detect specific antibodies in a large number of TT/TB cases may be due to the low antigenic load. Since studies in India have indicated that 74–90% of the early cases of tuberculoid leprosy may be of a self-healing nature ([7, 13]), an increase in the antibody level could be interpreted as a prognostic signal for the shifting of the disease toward the lepromatous pole of the spectrum.

In the group of household contacts of multibacillary leprosy cases, 46% showed antibody positivity, much higher than the reported incidence rate of leprosy. Abe's studies with school children of Okinawa, Japan, have also shown that the FLA-ABS test was positive at least 200 times the actual incidence rate of leprosy ([1]). Similar observations have been made in the study of leprosy contacts in Ethiopia ([16]). The positive lepromin skin test has also been found to be much more frequent in a leprosy endemic area than the actual incidence rate of leprosy ([11]). Presumably, the infection in contact subjects results in adequate protective immunity and a self-healing outcome ([8, 14]).

The much needed epidemiological test for leprosy should be highly specific and sensitive enough to detect early cases of the infectious form of leprosy ([24, 25]). The detection of such cases is mandatory for an epidemiological test if it is aimed at reducing the leprosy incidence rate. Therefore, the apparent restriction of the reported anti-MY2a SACT assay as compared to other tests with a selective detection of lepromatous cases is an apparent advantage. However, its prognostic capability to predict the development of lepromatous leprosy at a subclinical stage is yet to be explored in future long-term clinical trials.

Apart from its use in epidemiological studies of family contacts, the SACT assay could also be beneficial for monitoring the emergence of drug-resistant bacilli in response to therapy. Initial unpublished data suggest a steady decline of ML04-specific antibody levels with continuing chemotherapy in dapsone-responsive patients.

SUMMARY

A serological antibody competition test (SACT) using a murine anti-*Mycobacterium leprae*-specific monoclonal antibody (ML04) has been evaluated in 96 untreated leprosy patients and 128 control subjects. The test is based on the competitive inhibition by antibodies from human test sera of the binding of the specific ^{125}I-ML04 murine monoclonal antibody to *M. leprae* soluble antigen coated microtiter plates. Along the spectrum of the disease, SACT positivity was observed in 100% of LL-BL, 87.5% of BB, and 46.7% of the TT-BT groups of patients. Moreover, 46.4% of apparently healthy household family contacts of multibacillary leprosy cases were also antibody positive. Sera from 15 contacts of paucibacillary leprosy cases as well as from 85 various nonleprosy subjects (tuberculosis, autoimmune disease, cancer, and healthy) were all found to be negative.

These results satisfy the requirements of a leprosy-specific serodiagnostic test. The striking increase of antibody titer in all of the LL/BL patients indicates a potential prognostic utility of this test for the lepromatous shift of the disease. The observed

positivity in a fraction of healthy leprosy contacts still requires prospective evaluation of disease incidence in a larger test sample.

RESUMEN

Se evaluó una "prueba serológica de competencia por anticuerpo" usando un anticuerpo monoclonal murino específico contra *Mycobacterium leprae* (ML04) en 96 pacientes con lepra no tratada y en 128 sujetos control. En la prueba, los anticuerpos presentes en los sueros problema inhiben competitivamente la unión del anticuerpo monoclonal marcado ML04-I^{125} con el antígeno soluble derivado del *M. leprae.* El ensayo se hace en microplacas de plástico recubiertas con antígeno. Se encontró que el 100% de los pacientes con lepra LL-BL, el 85% de los BB, y el 46.7% de los TT-BT, dieron un resultado positivo en esta prueba. Además, el 46.4% de los contactos familiares (aparentemente sanos) de los pacientes leprosos multibacilares dieron también una prueba positiva. Los sueros de 15 contactos de pacientes paucibacilares y los sueros de 85 individuos sin lepra (con tuberculosis, con enfermedades autoinmunes, con cáncer o sanos) resultaron, todos, negativos.

Estos resultados satisfacen los requerimientos de una prueba serodiagnóstica específica para la lepra. El sorprendente incremento en el título de anticuerpos en todos los pacientes LL/BL, sugiere una utilidad pronóstica potencial de esta prueba.

La positividad observada en una fracción de los contactos aparentemente sanos, aún requiere de una evaluación prospectiva de la incidencia de la enfermedad en una muestra poblacional más grande.

RÉSUMÉ

Chez 96 malades de la lèpre non traités, et chez 128 sujets témoins, on a pratiqué des épreuves de compétition des anticorps du sérum (SACT), en utilisant un anticorps monoclonal murin spécifique contre *Mycobacterium leprae.* Cette épreuve est basée sur une inhibition compétitive de la liaison de l'anticorps monoclonal murin spécifique ^{125}I-ML04 à l'antigène soluble de *M. leprae* dont on revêt les plaques de microtitrage, par les anticorps provenant des échantillons de sérum humain que l'on étudie. Tout au long du spectre de la maladie, on a relevé des épreuves SACT positives chez 100% des malades LL-BL, 87,5% des malades BB, et 46,7% des malades appartenant aux groupes TT-BT. De plus, parmi des contacts familiaux apparemment en bonne santé partageant le foyer de malades atteints de lèpre multibacillaires, 46,7% présentait également une réaction d'anticorps positive. Par contre, les échantillons de sérum obtenus chez 15 contacts de malades paucibacillaires et chez 45 individus ne souffrant pas de lèpre, mais d'autres maladies telles que la tuberculose, des maladies auto-immunitaires, des cancers, ainsi que des sujets sains, se sont tous révélés négatifs.

Ces résultats satisfont les exigences d'une épreuve sérodiagnostique spécifique pour la lèpre. L'augmentation frappante du titre des anticorps chez tous les malades LL/BL indique que cette épreuve pourrait être fort utile pour prédire l'évolution de la maladie vers la forme lépromateuse. Le fait que certains des contacts sains de malade présentent une réaction positive mérite d'être étudié plus avant, en menant une étude longitudinale de l'incidence de la maladie dans un échantillon plus considérable d'individus.

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