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Sero-Immunoreactivity of Cloned Protein Antigens of *Mycobacterium leprae*[1]

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An important aspect in the epidemiologic study of leprosy is the lack of reliable and appropriate technology for the detection of subclinical infection in patients infected with *Mycobacterium leprae*. Leprosy is one of the major disabling diseases and imposes a considerable burden in terms of morbidity. The disease presents a remarkably "broad spectrum of clinical stages ranging from tuberculoid leprosy (TT) to a stage designated as lepromatous leprosy (LL). Patients with TT exhibit strong cell-mediated immune responses to *M. leprae,* a low level of circulating antibody, and low levels of bacilli (paucibacillary) in their lesions ([5, 15]). By contrast, most LL patients exhibit a high level of humoral response but fail to display specific cell-mediated responsiveness to *M. leprae,* and exhibit an abundant number of bacilli (multibacillary) in the lesions ([5, 15]).

Our main objective was to screen several molecularly cloned protein antigens (12-kDa, 18-kDa, 28-kDa, 36-kDa and 65-kDa) of *M. leprae* with sera from leprosy patients to determine if there is a specific distribution of antibodies to each of the protein antigens according to the clinical and bacteriological spectrum of the disease. This would permit the recognition of a possible serodiagnostic and/or seroprognostic test which could be used in an epidemiological survey. All sera were screened by indirect enzyme-linked immunosorbent assay (ELISA).

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MATERIALS AND METHODS

Sera. Sera from leprosy patients in various stages of infection and their contacts were used for the study. The time course of the disease was similar in all patients screened. The patients had been clinically and histologically classified according to the Ridley-Jopling scheme ([19]) prior to the collection of the sera. Some of the serum samples were collected from patients undergoing extensive chemotherapy. The sera were labeled accordingly, aliquoted, and maintained at −20°C. Sera from normal healthy individuals (NHS) and from contacts of leprosy patients (randomly chosen from an endemic area) were similarly maintained. These controls were not age and sex matched with the cases.

Preparation of *M. leprae* cell sonicate. Purified armadillo-derived *M. leprae* cells (obtained from Dr. P. J. Brennan and Dr. S. Hunter, Colorado State University, Fort Collins, Colorado, U.S.A.) were washed three times in 0.05% saline and disrupted by sonication at 4°C for 12 min using a W-375 sonicator (Heat System--Ultrasonics, Plainview, New York, U.S.A.) with a 100 w energy output. Thereafter, the supernate, separated by centrifugation, was aliquoted and stored at −20°C after the addition of phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Co., St. Louis, Missouri, U.S.A.).

Preparation of crude lysate from lysogens. Lysogens of λgt11 with different gene (encoding *M. leprae* 12 kDa, 18 kDa, 28 kDa, 36 kDa, and 65 kDa) inserts maintained in *Escherichia coli* Y1089 were obtained from Dr. Richard Young, Massachusetts Institute of Technology, Cambridge, Massachusetts, U.S.A.) and λgt11 phage (without insert) maintained in *E. coli* Y1090 was obtained from Dr. J. Clark-Curtiss (Washington University, St. Louis, Missouri,

U.S.A.). The lysogens obtained were as follows: Y3184–λgt11 recombinant encoding *M. leprae* 12-kDa determinant; Y3179–λgt11 recombinant encoding an *M. leprae* 18-kDa antigen determinant; Y3164–λgt11 recombinant encoding an *M. leprae* 28-kDa antigen determinant; Y3180–λgt11 recombinant encoding an *M. leprae* 36-kDa antigen determinant; and Y3178–λgt11 recombinant encoding an *M. leprae* 65-kDa antigen determinant.

A single colony of each lysogen was inoculated in 18 ml of Luria broth (LB; pH 7.5) containing 50 μg/ml ampicillin (added only for Y3179 and Y3178, since Y3184, Y3164, and Y3180 do not grow in the presence of ampicillin), and incubated overnight at 32°C in a shaker incubator. On the following day, 100 μl of the overnight culture was inoculated in 20 ml of LB containing 0.2% maltose. The cultures were grown with vigorous shaking in a waterbath maintained at 32°C until an optical density (OD) of 0.5 was obtained at 595 nm. The lysogen was induced by shifting the temperature from 32°C to 45°C for a period of 20 min with vigorous shaking. Subsequently, isopropyl β-D-thiogalactopyranoside (IPTG; 10 mM) was added, and the culture was incubated for 1 hr at 38°C. The intact cells were centrifuged and resuspended in phosphate buffered saline (PBS) or sodium dodecyl sulfate (SDS) buffer and stored at −80°C. Complete lysis of the cells was performed by freeze-thaw cycles as required. Each lysate was checked for the presence of *M. leprae* protein by immunoblotting (with the use of monoclonal antibody) and then used for further studies.

Monoclonal antibodies to *M. leprae* proteins. Monoclonal antibodies to each *M. leprae* protein (MC2404 for 65 kDa, MC5828 for 36 kDa, MC4742 for 28 kDa, MC8026 for 18 kDa, and MC8909 for 12 kDa) [7] were obtained from WHO IMMLEP, Geneva, Switzerland, through Dr. T. Shinnick (Centers for Disease Control, Atlanta, Georgia, U.S.A.).

Polyclonal antiserum to *M. leprae* cell sonicate. New Zealand white male rabbits were used for raising a hyperimmune antiserum to *M. leprae* cell sonicate. Three rabbits were used for this purpose. Prior to immunization, the animals were ear-bled to collect normal rabbit serum. Each rabbit was administered 1 ml of a 1:1 (v/v) mixture of the *M. leprae* cell sonicate and Freund's incomplete adjuvant (Difco Laboratories, Detroit, Michigan, U.S.A.) subcutaneously in the hind quarter. Six such injections were given spaced 1 week apart. This has been the established protocol in our laboratory for raising a strong hyperimmune serum. One week after the last injection, each animal was ear-bled, and the serum was analyzed by the immunodiffusion method [9, 18] to determine the antibody response. Since the response of each rabbit was similar, the animals were exsanguinated in the eighth week after the first injection and the serum samples were pooled, aliquoted, and stored at −20°C.

Indirect ELISA. Polystyrene, 96-well, flat-bottom microtiter plates (Falcon; Becton Dickinson Labware, Lincoln Park, New Jersey, U.S.A.) were coated with 100 μl of each lysate (containing *M. leprae* protein) per well for 3 hr at 37°C. Each lysate was initially diluted 1:100 in carbonate bicarbonate buffer (pH 8.6) prior to use. *M. leprae* cell sonicate was also used as the coating antigen. The plates were then washed six times with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 100 μl of 15% bovine serum albumin (BSA) in PBS at room temperature for 1 hr. The plates were washed once in PBS-T and stored overnight at 4°C. On the following day, the wells were filled with 100 μl of test and control serum diluted 1:200 in Tris-buffered saline (TBS) and incubated at room temperature for 2 hr. Washing was followed by incubating with 100 μl of 1:1000 diluted peroxidase-conjugated rabbit anti-human polyvalent immunoglobulin (Sigma) at room temperature for 1 hr. Subsequently, after washing six times with PBS-T, each well was incubated with 100 μl of substrate solution (10 mg orthophenylene diamine dihydrochloride in 1 ml methanol mixed with 99 ml distilled water and 0.1 ml of 3% hydrogen peroxide) at room temperature for 30 min. The color development was stopped by the addition of 50 μl of 4 N H_2SO_4 per well, and the OD was measured after 15 min in a Titertek Multiskan reader using a 492 nm filter. All of the serum samples were tested in triplicate, and the mean OD was recorded for each sample. A positive reaction was defined as the OD which was greater than the

mean control (NHS) value plus four standard deviations.

RESULTS

In this study, 225 serum samples were tested with cloned *M. leprae* antigens, while 93 serum samples were tested with *M. leprae* cell sonicate using an ELISA. Most serum samples from multibacillary patients (BL and LL), irrespective of the type and duration of treatment, reacted strongly with the 65-, 36- and 28-kDa lysates as well as with *M. leprae* cell sonicate (The Table). Reactivity of the samples gradually decreased toward the tuberculoid end of the spectrum. However, a small number of the sera also showed positive reactions with the 18- and 12-kDa lysates. Only one serum sample collected from a contact showed positive reaction with 65- and 12-kDa. Most of the control sera (NHS) showed minimal reaction with both the cloned antigens and with *M. leprae* cell sonicate. No reaction was observed when the sera were tested with the control lysate.

DISCUSSION

The present study gives the data on the reactivity of leprosy sera at all stages of infection with the *M. leprae* cell sonicate and the crude lysates of the cloned proteins by ELISA. As stated in the Methods section, the criterion for defining positive values in ELISA was chosen so as to ensure the exclusion of doubtful and false-positive results in the test. The results obtained indicate that the majority of the sera from multibacillary patients recognize the 65-, 36- and 28-kDa antigens to a high degree (93.1%). On the other hand, sera from the paucibacillary patients exhibited a considerably lower degree of reactivity (16%) to the same antigens. Whether the patients received treatment or were untreated did not influence the reactivity of the sera. Most of the serum samples showed no remarkable binding to either the 18- or 12-kDa lysates, indicating that these antigens are probably less immunoreactive than the other three. It is possible that if the antibodies to these antigens are present, they may be at a lower concentration and were undetectable by the method used. It should also be noted that

THE TABLE. *Enzyme-linked immunosorbent assay: reaction of various sera from leprosy patients, contacts and normal healthy individuals with cloned protein lysates and cell sonicate of* M. leprae.

Type	65 kDa		36 kDa		28 kDa		18 kDa		12 kDa		*M. leprae* cell sonicate	
	No. positive[a]/total	Mean OD	No. positive/total	Mean OD	No. positive/total	Mean OD	No. positive/total	Mean OD	No. positive/total	Mean OD	No. positive/total	Mean OD
Leprosy												
LL	55/60	0.99 (0.24)[b]	55/60	0.91 (0.25)	59/60	0.94 (0.25)	11/60	0.44 (0.19)	4/60	0.40 (0.19)	28/30	1.29 (0.17)
BL	40/45	0.84 (0.21)	41/45	0.89 (0.18)	60/60	0.75 (0.23)	8/50	0.43 (0.16)	5/45	0.35 (0.19)	16/20	0.91 (0.18)
BB	12/25	0.69 (0.24)	10/25	0.52 (0.21)	8/25	0.32 (0.20)	2/12	0.41 (0.23)	1/25	0.32 (0.19)	ND[c]	ND
TT/BT	8/43	0.40 (0.26)	6/43	0.47 (0.20)	4/43	0.34 (0.12)	2/43	0.40 (0.14)	2/43	0.40 (0.13)	8/32	0.42 (0.22)
Contacts	1/12	0.22 (0.07)	0/12	0.22 (0.03)	0/12	0.27 (0.09)	0/12	0.35 (0.11)	1/12	0.23 (0.07)	0/5	0.37 (0.65)
Normal human serum	0/40	0.52 (0.06)	0/40	0.43 (0.05)	0/40	0.43 (0.06)	0/40	0.40 (0.05)	0/40	0.41 (0.04)	0/6	0.51 (0.06)

[a] Positive values were defined with OD greater than the control mean plus 4 standard deviations.
[b] Numbers in the parentheses indicate the standard deviation for each group.
[c] ND = Not done.

the criteria for a positive test are vigorous, which may explain the relative lack of responses in paucibacillary patients.

Our data with regard to the seroreactivity of the different cloned proteins are in agreement with those of other investigators who have shown the presence of circulating antibodies in sera from leprosy patients in various stages of infection. Levis, *et al.* ([14]) have shown higher levels of antibodies to these antigens (especially the 65-kDa) in LL and BL patients as compared to TT or BT patients. Klatser, *et al.* ([12]) have reported that LL patients show high reactivity to all the antigens ranging from 12-kDa to 86-kDa. Similar observations with regard to the high degree of seroreactivity in multibacillary patients as opposed to paucibacillary patients have been made by Sathish, *et al.* ([20]) and Cherayil, *et al.* ([3]). Considering the seroreactivity of each individual protein, it is evident that the 65-kDa appears to be the most active component of the known antigenic mosaic of *M. leprae* in multibacillary leprosy, followed by the 36-kDa. The least reactive component appears to be the 12-kDa, antibodies to which are present in the leprosy sera only to a minimum degree. The detection of circulating antibodies appears to be directly dependent upon the clinical status of the patients, with the LL and BL cases showing more humoral response than the TT and BT cases. Klatser, *et al.* ([11]) have shown that a large proportion of sera from patients in the TT or BT part of the spectrum react with the 36-kDa antigen. This appears to be in contrast to our observation and the observations of several others. Ehrenberg and Gebre ([6]) have reported that most of the sera from BT patients bind to *M. leprae* proteins greater than 70-kDa. Vega-Lopez, *et al.* ([21]) have reported the recognition of five proteins (33-, 25-, 18-, 15- and 12-kDa) by a majority of LL sera using Western blotting; whereas Chakrabarty, *et al.* ([2]) have shown the recognition of only two proteins (33- and 12-kDa) by this method.

That sera from multibacillary leprosy patients possess a very high level of circulating antibodies as opposed to those from paucibacillary leprosy patients has been well documented. Navalkar, *et al.* ([16, 17]) first demonstrated this phenomenon when they studied the antibody levels in sera from patients in different stages of infection, using various mycobacterial antigens. Subsequently, Convit, *et al.* ([4]) and Kwapinski, *et al.* ([13]) confirmed these observations. Our observations with regard to the responsiveness of sera from intrafamilial contacts are not in total agreement with the observations made by other investigators. The lower positivity observed with contacts could be explained by the fact that the criteria chosen for a positive test in this study are vigorous. Amezcua, *et al.* ([1]) have reported a high percentage of seropositivity in household contacts of Mexican leprosy patients, while Gonzalez-Abreu, *et al.* ([8]) have observed an overall positivity of less than 10% with sera from contacts. Hartskeerl, *et al.* have reported the characterization of recombinant clones that produce *M. leprae* antigens which were recognized by sera from contacts of leprosy patients ([10]). However, none of the recombinant antigens recognized by the contacts' sera were identical or related to the well-characterized antigens of *M. leprae* used in this study.

Our results, in general, differ from the observations made by other investigators. It is likely that factors such as a) the clinical and bacteriological status of the patients evaluated, b) the source of sera, and c) the methodologies employed by individual investigators could possibly be attributed to the variability of results emanating from different laboratories. In this study, we have made an effort to identify antibodies not only against the *M. leprae* cell sonicate but also against the various cloned proteins of *M. leprae.* A common fact that emerges is that multibacillary leprosy patients, treated or untreated, exhibit a high degree of reactivity to the different antigenic preparations used as compared to the paucibacillary or bacillary-negative patients. It is, however, important to note that for the selection of an appropriate antigen for leprosy diagnosis, it is imperative to study a larger number of healthy controls, contacts and also other pathological sera from patients with mycobacterial infections that are prevalent in an endemic environment. Unavailability of such materials in greater numbers permitted us to screen only those samples that were made available to us.

SUMMARY

Sera from 173 leprosy patients with various types of disease (tuberculoid = TT, borderline tuberculoid = BT, borderline lepromatous = BL, and lepromatous = LL), 12 intrafamilial contacts, and 40 normal healthy individuals were assayed in an indirect enzyme-linked immunosorbent assay (ELISA) using *Mycobacterium leprae* antigens. Recombinant clones carrying *M. leprae* antigens, namely, Y3184 (12 kDa), Y3179 (18 kDa), Y3164 (28 kDa), Y3180 (36 kDa), and Y3178 (65 kDa) and a cell sonicate from armadillo-derived *M. leprae* were used for the study. A high degree of reactivity with the 65-kDa, 36-kDa, and 28-kDa protein lysates was observed in most of the sera from multibacillary patients, with a low degree of positivity with 18 kDa and 12 kDa. Only a few sera from paucibacillary patients showed positive reactions. The majority of the contacts' sera tested showed no reactivity with these antigens.

RESUMEN

Usando un inmunoensayo enzimático (ELISA) con antígenos de *Mycobacterium leprae* se analizaron los sueros de 173 pacientes con diferentes tipos de lepra (tuberculoide, TT; tuberculoide subpolar, BT; lepromatosa subpolar, BL; y lepromatosa, LL), los sueros de 12 contactos familiares y los de 40 individuos sanos no relacionados. Para el estudio se usaron las siguientes clonas recombinantes portadoras de antígenos del *M. leprae*; Y3184 (12 kDa), Y3179 (18 kDa), Y3164 (28 kDa), Y3180 (36 kDa), y Y3178 (65 kDa), además de un sonicado de *M. leprae* cultivado en armadillo. En la mayoría de los pacientes con lepra multibacilar se observó un alto grado de reactividad con los lisados conteniendo las proteínas 65 kDa, 36 kDa, y 28 kDa, y un bajo grado de reactividad con las proteínas 18 kDa y 12 kDa. Sólo unos cuantos sueros de los pacientes paucibacilares mostraron reacciones positivas. La mayoría de los sueros de los contactos probados no mostraron reactividad con estos antígenos.

RÉSUMÉ

Les sérums de 173 patients lépreux présentant différents types de la maladie (tuberculoïde = TT, borderline tuberculoide = BT, borderline lépromateux = BL et lépromateux = LL), 12 contacts intrafamiliaux, et 40 individus normaux en bonne santé ont été titrés par un test enzymatique indirect (ELISA) utilisant des antigènes de *Mycobacterium leprae.* Des clones recombinants porteurs des antigènes de *M. leprae* Y3184 (12 kDa), Y3179 (18 kDa), Y3164 (28 kDa), Y3180 (36 kDa), et Y3178 (65 kDa) et un sonicat cellulaire de *M. leprae* de tatou ont été utilisés pour cette étude. Un haut degré de réactivité a été observé avec les protéines de 65-kDa, 36-kDa et 28-kDa dans la majorité des sérums de patients multibacillaires, et un degré faible de positivité pour les protéines de 18 kDa et 12 kDa. Seuls quelques sérums de patients paucibacillaires ont montré des réactions positives. La majorité des sérums de contacts n'a montré aucune réaction avec ces antigènes.

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