ARTICLE

Specific IgG antibody responses may be used to monitor leprosy treatment efficacy and as recurrence prognostic markers

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Abstract Although curable, leprosy requires better diagnostic and prognostic tools to accompany therapeutic strategies. We evaluated the serum samples of leprosy patients from Venezuela and Brazil for reactivity against the specific recombinant proteins, ML0405 and ML2331, and the LID-1 fusion protein that incorporates both of these antigens. Antigen-specific IgG was highest in lepromatous leprosy patients (LL) and decreased across the disease spectrum, such that only a small subset of true tuberculoid patients (TT) tested positive. The impact of multidrug therapy (MDT) on these antibody responses was also examined. Several years after treatment, the vast majority of Venezuelan patients did not possess circulating anti-LID-1, anti-ML0405, and anti-ML2331 IgG, and the seropositivity of the remaining cases could be attributed to irregular treatment. At discharge, the magnitude and proportion of positive responses of Brazilian patients against the proteins and phenolic glycolipid (PGL)-I were lower for most of the clinical forms. The monthly examination of IgG levels in LL patient sera after MDT initiation indicated that these responses are significantly reduced during treatment. Thus, responses against these antigens positively correlate with bacillary load, clinical forms, and operational classification at diagnosis. Our data indicate that these responses could be employed as an auxiliary tool for the assessment of treatment efficacy and disease relapse.

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Abbreviations

BB	Borderline borderline
BI	Bacterial index
BL	Borderline lepromatous
BT	Borderline tuberculoid
C	Control
EC	Endemic control
HHC	Healthy household contact
LI	Leprosy indeterminate
LID	Leprosy Infectious Disease Research Institute (IDRI) diagnostic
LL	Lepromatous leprosy
MB	Multibacillary
MDT	Multidrug therapy

NEC	Non-endemic control
PB	Paucibacillary
PGL	Phenolic glycolipid
TT	True tuberculoid

Introduction

Leprosy is a devastating human disease caused by *Mycobacterium leprae* infection. Leprosy presents a variety of manifestations characterized by clinical, histopathological, and immunological evaluations, which can be classified into five clinical forms: lepromatous leprosy (LL), borderline lepromatous (BL), mid-borderline (BB), borderline tuberculoid (BT), and tuberculoid (TT) [1]. For treatment purposes, patients are categorized as multibacillary (MB; encompassing LL, BL, BB, and some BT) and paucibacillary (PB; encompassing TT and some BT). At the extreme MB pole, in the absence of a strong cellular immune response, LL patients do not control bacterial replication and have high bacterial indices (BI) [2]. Infection is disseminated and patients classically present with multiple, large skin lesions. In marked contrast, at the extreme PB pole, TT patients demonstrate a specific cell-mediated immunity against *M. leprae* and have a low BI. PB leprosy patients classically present with five or less focal lesions.

The implementation of World Health Organization (WHO)-provided multidrug therapy (MDT) for widespread, worldwide treatment has resulted in the drastic reduction of registered leprosy cases from approximately 12 million reported in 1985 to less than 250,000 reported in 2006 [3]. The worldwide annual rate of new case detection for leprosy appears to have stabilized at approximately 250,000 over the last few years [3]. Outside India, however, the annual number of new leprosy cases has remained stable for a longer period and has recently increased in some countries. Mathematical modeling suggests that the disease will remain a major public health problem for at least several decades [4].

Although advances in leprosy surveillance and case management have been made, measures to assess treatment efficacy to facilitate the early recognition of treatment failure are still needed. While MDT remains effective in the majority of cases, this efficacy will be diminished by the development of drug resistance. Over the last few years, there have been an increasing number of reports documenting drug-resistant *M. leprae* strains [5–9]. Patients can be treated for extended periods of time before it is realized that treatment is having no impact. The widespread emergence of drug-resistant *M. leprae* could have catastrophic consequences, undoing the efforts of the last 20 years and

causing a rebound in leprosy incidence. This is particularly critical because there are very few alternative treatments currently available and the identification of new treatments is hampered by the length of time currently required for assessment. Simple and objective measures of treatment could facilitate both the earlier recognition of drug resistance and the identification of alternative treatments.

We have recently identified several protein antigens that are specifically recognized by leprosy patients [10–13]. The aim of this study was to evaluate antigen-specific antibody responses during standard leprosy treatment in order to determine if they can be used as simple indicators of successful treatment. We analyzed the antibody response against recently identified protein antigens to determine if these were changed after and during treatment.

Materials and methods

Patient samples

Patients were initially classified as MB and PB leprosy by clinical examination. When possible, patients were then fully categorized within the classification of the Ridley–Jopling scale by clinical and histological observations carried out by qualified personnel (bacterial index, skin lesions, nerve involvement, and histopathology). To serve as controls, healthy contacts and individuals with no known contact with leprosy patients were also recruited. Patient and control sera were collected at the following sites, according to the following guidelines:

– Venezuela. Newly diagnosed patients were recruited at the Central Service of Dermatology, Institute of Biomedicine, Caracas (44 LL, 28 BL, 13 BB, 19 BT, 2 TT, 6 IL, and 15 controls). Former patients (*n*=57; 27 MB (1 LL, 9 BL, 2 BB, and 15 not histologically defined), 25 PB (11 BT, 6 TT, and 8 not histologically defined), 5 LI [leprosy indeterminate]), having undergone treatment approximately 10 years earlier (1999–2002) with MDT regimen of 6 months for PB or 2 years for MB leprosy, were recruited in Venezuelan villages within leprosy hyperendemic regions. EC (*n*=29) and contacts (*n*=51) were also recruited from within these villages.

– Uberlândia, Brazil. Serum samples of newly diagnosed leprosy patients (*n*=107; 23 LL, 14 BL, 19 BB, 19 BT [MB], 15 BT [PB], and 17 TT) and household contacts (*n*=200) recruited at the National Reference Center of Leprosy and Sanitary Dermatology of the Clinics' Hospital, Federal University of Uberlândia (CRE-DESH/CHU/UFU) under the Federal University of Uberlândia Ethics Committee approval number 025/

2000. Patients received an operational classification as PB or MB for treatment purposes, based on lesion characteristics, bacterial index, and PGL-I serology. TT forms or BT forms with five or less than five skin lesions and negative BI were considered to be PB. BT forms with more than five skin lesions and/or a BI from zero to two in the skin lesion were considered to be MB [14]. Sera were collected at diagnosis and at the end of MDT.

– São Paulo, Brazil. Newly diagnosed patients (*n*=20; 12 MB [5 LL, 7 BL] and 8 PB) were recruited at the São Paulo Center for Dermatology, São Paulo, Brazil. Sera were collected at the time of initial diagnosis, monthly during treatment, and then again at the end of complete MDT.

Antibody ELISA

Serum antibodies to the *M. leprae* antigens were monitored by enzyme-linked immunosorbent assay (ELISA). Anti-recombinant protein detection ELISA was conducted by coating 96-well microtiter plates (Polysorp®, Nunc, Rochester, NY) with 1 μg/ml protein or 200 ng/ml NDO-BSA (the synthetically derived B-cell epitope of PGL-I conjugated to BSA; kindly supplied by Dr. John Spencer, Colorado State University, under NIH contract N01 AI-25469), in bicarbonate buffer overnight at 4°C. The plates were then blocked for 1 h at room temperature with PBST with 1% BSA on a plate shaker. Serum diluted appropriately in 0.1% BSA was added to each well, and the plates were incubated at room temperature for 2 h with shaking. The plates were washed with buffer only, then horseradish peroxidase-conjugated IgG or IgM (Rockland Immunochemicals, Gilbertsville, PA), diluted in 0.1% BSA, was added to each well and incubated at room temperature for 1 h with shaking. After washing, the plates were developed with peroxidase color substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD), and the reaction quenched by the addition of 1 N H_2SO_4. The optical density of each well was read at 450 nm.

Anti-PGL-I antibody detection ELISA was performed in 96-well microtiter plates (Maxisorp®, Nunc), which were coated with 50 μL of native PGL-I (kindly supplied by Dr. John Spencer, Colorado State University) diluted in absolute ethyl alcohol. The plates were then blocked with BSA 1% for 1 h at 37°C, and washed with PBS. Serum samples were added in duplicate using a dilution of 1:100 1%, BSA/PBS, and incubated for 1 hr at 37°C, followed by washing. The anti-human IgM-peroxidase conjugate (Sigma Chemical Co., St. Louis, MO) was added to the plates at a dilution of 1:10,000 in BSA 1%, again for 1 h at 37°C. After a series of PBS washes, the o-phenylenediamine dihydrochloride (OPD, Sigma) enzyme substrate was added

to the plates and incubated at room temperature for 5 min in the dark. The reaction was stopped by the addition of 25 μL of H_2SO_4 4N. The optical density (OD) was obtained using a microplate reader at 492 nm (Thermo Plate, TP-Reader, Rayto Life and Analytical Sciences Co. Ltd, Germany). The ELISA results were analyzed based on the calculation of ELISA indices, a procedure employed when the antibody target is not present in every sample, and negative values are used to normalize data in different assays and to reduce inter-test variations. The calculation of cut-off values was performed by adding four standard deviations (4 SDs) on top of the mean OD of three blanks (no sample) and three negative control samples per plate, which was set to cover a 99.99% confidence interval. Negative samples were previously established by using individuals obtained from a non-endemic region, with no history of leprosy, and with negative polymerase chain reaction (PCR) result (blood, skin smears, oral and nasal swabs) and negative serum anti-PGL-I. Two known positive controls were also used in each plate for verification purposes after normalization of the data. If the coefficient of variation for positive controls was greater than 2%, the assay was considered to be inadequate and it was repeated. The antibody titers were expressed as the ELISA index (EI) according to the following formula: $EI = OD_{sample}/OD_{cut-off}$, as described previously [15]. EI values above 1.1 were considered to be positive.

Results

Antibody responses to proteins correlate with the clinical form

We recently identified potent and highly specific antibody responses against several protein antigens in serum from MB leprosy patients. As the magnitude of anti-PGL-I (or NDO-BSA) IgM responses correlate with clinical forms, we analyzed the response of patients that were fully characterized across the Ridley–Jopling scale. The median antibody responses were highest in lepromatous LL patients, slightly lower in BL patients, and continued to be reduced as the clinical form indicated lower BI (Fig. 1). In these analyses, using a threshold of ELISA index above 1.1, 97.7% of LL patients, 96.4% of BL patients, and 76.9% of BB patients were positive for anti-LID-1 responses, with 90.9%, 85.7%, and 38.5%, respectively, having ELISA indices above 5. These results support the use of this chimeric fusion protein for the diagnosis of MB leprosy.

Negligible antibody responses after treatment

It has previously been demonstrated that anti-NDO-BSA IgM responses wane after treatment [16–18]. To determine
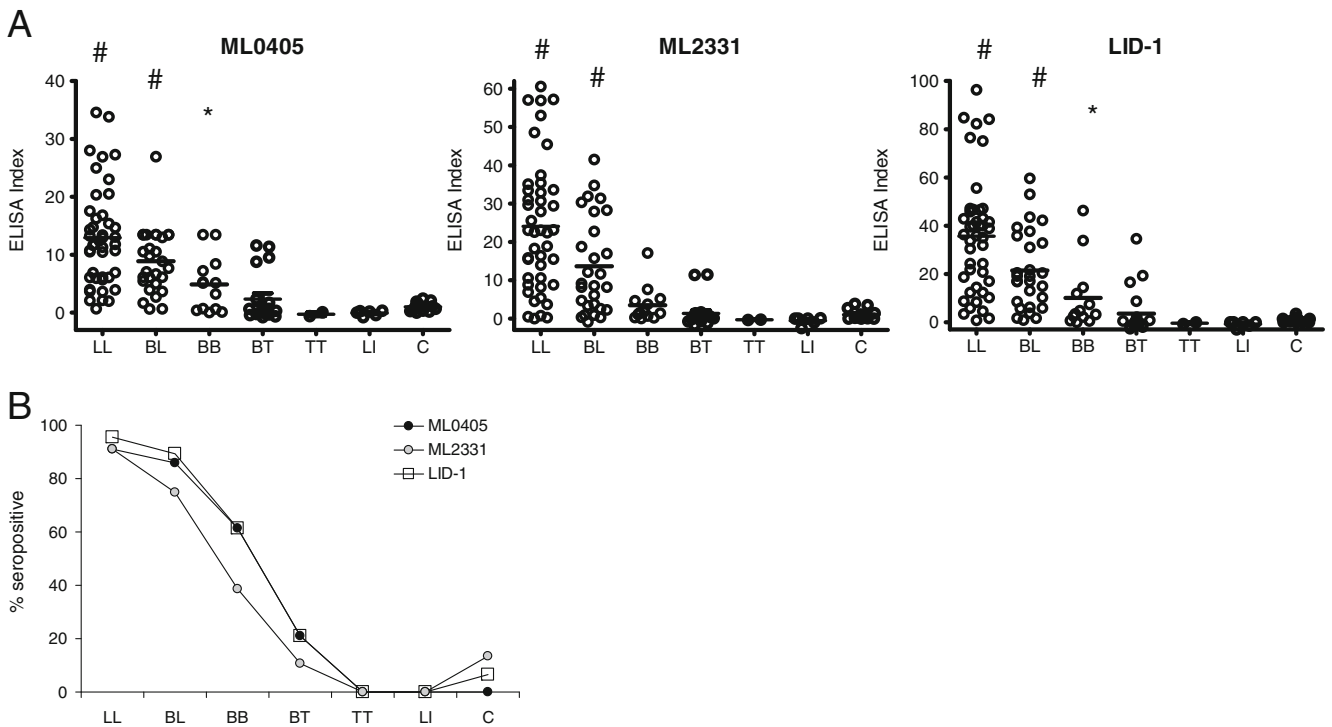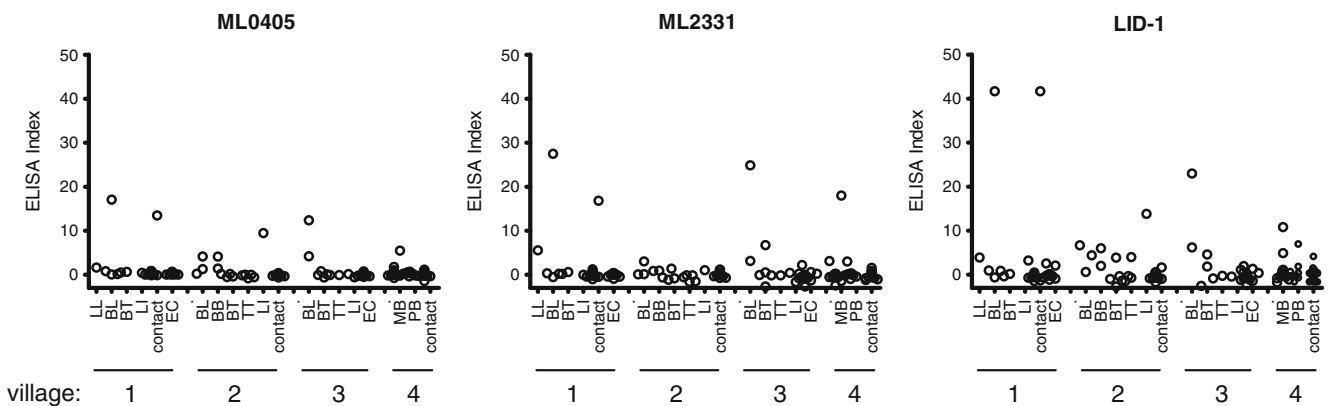
Fig. 1 Antibody responses of leprosy patients. Sera from Venezuelan leprosy patients, who were fully characterized to permit placement into the Ridley–Jopling scale, were assessed by enzyme-linked immunosorbent assay (ELISA) against ML0405, ML2331, and LID-1. Protein reactivity was assessed by IgG binding. In **a**, each point represents the ELISA index of an individual serum and the median is represented by a *line*. *=$p<0.05$ and #=$p<0.001$ versus control (C). In **b**, the percentage of positive responders within each histologically defined leprosy category is plotted

if antibodies against proteins were similarly affected by treatment, we analyzed the response of individuals who had been provided MDT several years before serum collection. Sera were collected from former patients, contacts, and controls within villages in Venezuela, where leprosy was considered to be endemic only a decade ago. Of the former leprosy patient sera analyzed, the majority had extremely low antibody responses to each protein that were not different from the control values (Fig. 2). Four of the 57

former patients exhibited responses that were interpreted as being positive compared to controls. Upon review, three of these individuals had previously been provided MDT for MB leprosy, but have received irregular treatment. The other former patient that tested positive by anti-LID-1 ELISA had been characterized as an indeterminate case and had been provided the shorter course of MDT intended for PB leprosy. One of the 90 contacts tested positive within these ELISA, and was subsequently determined to have

Fig. 2 Treatment clears antigen-specific antibody responses among leprosy patients. Sera from previously treated leprosy patients and untreated contacts from four leprosy-endemic villages in Venezuela were analyzed by ELISA. Each point represents the ELISA index obtained with an individual serum

sub-clinical infection. These data and clinical information indicate that positive responses to these proteins are indicative of active leprosy and that responses may disappear upon successful treatment; therefore, antibody response monitoring should be maintained during treatment in order to define the time of discharge.

Pre- and post-treatment antibody responses

To expand this observation, we then compared the presence of anti-PGL-I IgM and anti-ML0405, ML2331, and LID-1 IgG responses of sera collected from 107 Brazilian patients across the leprosy spectrum at the initial diagnosis and again after treatment, as well as 200 healthy household contacts (HHC). PGL-I and the LID-1 chimeric antigen, as well as its components ML0405 and ML2331, all readily detected patients with high bacterial burdens (LL; Fig. 3a). Recognition decreased across the leprosy spectrum, such that few patients with low bacterial burdens had detectable antibodies (TT; Fig. 3a). PGL-I and LID-1 were each detected by 11.5% of HHC sera, while the individual components of LID-1 were, surprisingly, detected by a greater proportion (36.5% for ML0405 and 19.5% for ML2331). At the time of clinical diagnosis, the cumulative proportion of patients across the spectrum displaying positive responses against LID-1, ML0405, ML2331, and PGL-I in this sampling was 67%, 62%, 65%, and 76%, respectively (Fig. 3). A combination of the LID-1 and PGL-I antigens gave a positive rate of 80% among all patients. These results are consistent with our findings in Venezuela.

In this study group, we also examined how treatment alters the antibody response by comparing the magnitude and percentage of positive responses against each antigen at the end of a modified standard WHO MDT (6 months for PB; 12 months for most MB forms, with the exception of a 24-month treatment for LL). For all antigens tested, with the exception of TT patients that already had low ELISA indices at diagnosis, there was a decrease in the ELISA indices after treatment (Fig. 3b). In parallel, a lower percentage of positive responses were observed at the end of treatment, with the exception of those patients that had the highest (LL) and the lowest (TT) bacterial burdens at intake (Table 1). These data indicate that antibody responses are lower at the end of treatment and suggest that these could be used to assess treatment efficacy.

Antigen-specific antibody responses decline during MDT

Finally, to determine the rate of decline of antigen-specific antibody responses, we analyzed sera collected from patients at regular intervals during early treatment. Patients were identified and recruited in São Paulo, Brazil, provided standard MDT, and the anti-ML protein responses were examined. As expected, lepromatous patients (LL and BL) had high and readily detectable antibody responses at the time of clinical diagnosis, while tuberculoid patients (BT and TT) had responses only marginally above those of non-endemic controls (NEC; Fig. 4). To provide a clearer picture of how the antibody responses were affected during MDT, we normalized the responses of each LL patient against their initial ELISA value for each antigen. It was evident that, for each patient, the anti-protein responses gradually declined throughout treatment (Table 2). While the anti-NDO-BSA response had declined an average of only 1% and the anti-ML0405 and anti-LID-1 responses had not significantly declined by the second month of treatment, the anti-ML2331 response was significantly reduced (Table 2). By 3 months of treatment, all of the anti-protein responses were significantly reduced, and by 5 months after the initiation of treatment, while the anti-NDO-BSA response had declined 10%, the anti-protein responses had declined approximately 30%. These data further suggest that the reduction of IgG antibodies against protein antigens could serve as an indicator of treatment efficacy.

Discussion

Clinical examination and bacterial index analysis remain the standard diagnostic method for leprosy, which limits the ability to conduct large-scale screening programs aimed at providing treatment to *M. leprae*-infected individuals in the early stages of disease development. Evaluating the success of such programs is further complicated by the need for follow-up clinical examinations in the absence of simpler endpoints. Our data indicate that protein antigens can provide a diagnosis of MB leprosy patients, and, similar to anti-PGL-I responses, these responses are highest in the LL form and decline across the spectrum toward the TT form. The majority of former patients lack circulating antibodies to the proteins analyzed, indicating that the antigen-specific antibodies do not persist, and, therefore, should not interfere with the diagnosis of relapse or re-infection. Finally, the protein-specific IgG responses were found to decline more rapidly than anti-PGL-I (or NDO-BSA) IgM responses, suggesting that they could be used to assess treatment efficacy.

As worldwide leprosy case numbers have dwindled, so have the number of trained leprologists. This has inadvertently increased the likelihood that clinical diagnosis is delayed or even missed, especially in regions where leprosy incidence is low [19–21]. The presence of elevated titers of anti-PGL-I IgM reflects the total bacterial load in the body; these antibodies, however, are generally low or absent in PB patients. We assessed antibody responses against a
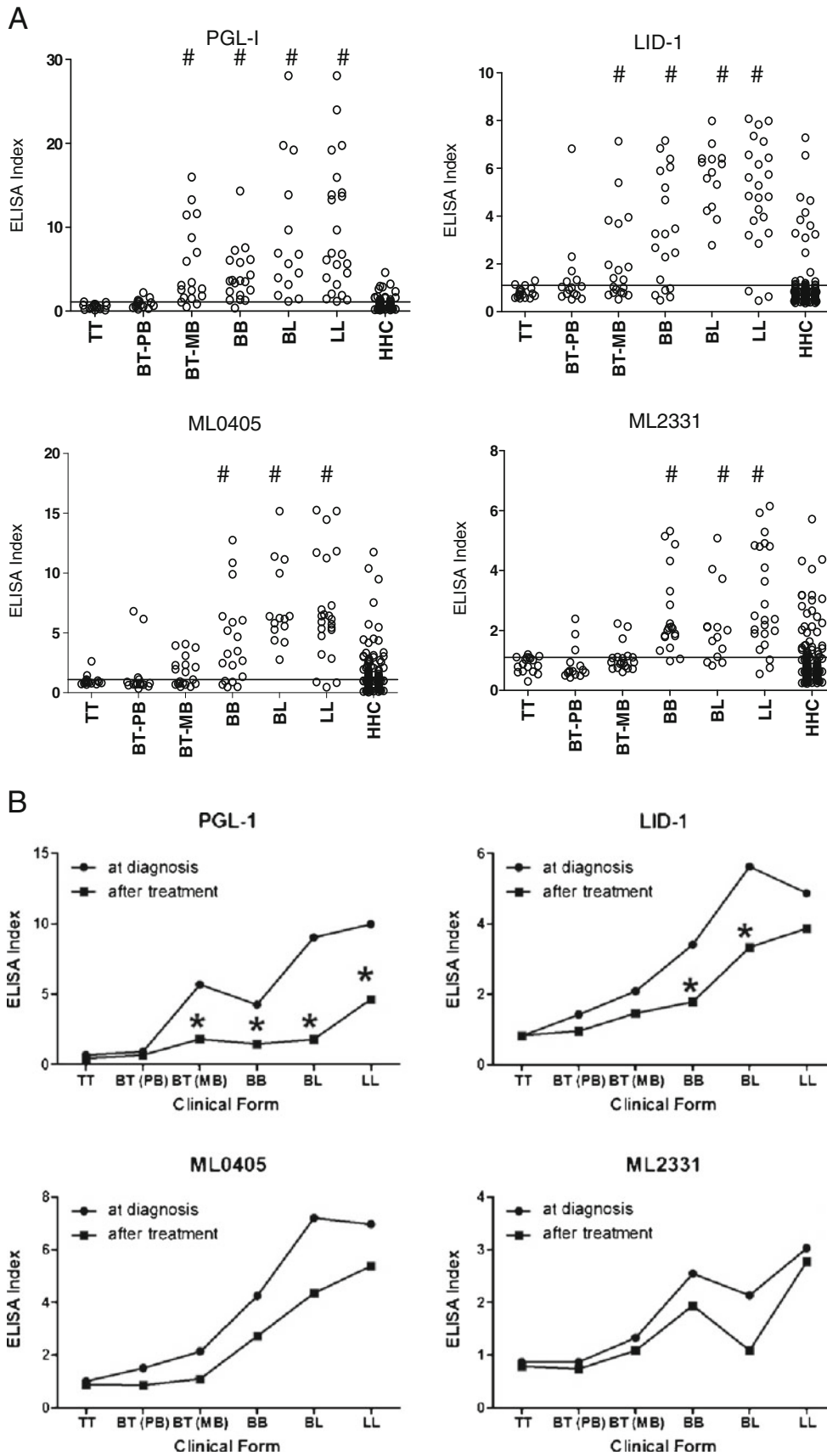
◀ **Fig. 3** Reduced numbers of positive antigen-specific antibody responses at the completion of multidrug therapy (MDT). Sera were collected from Brazilian patients across the leprosy spectrum (LL, BL, BB, BT, and TT) at the beginning and end of treatment, and antibody presence determined by ELISA. PGL-I and recombinant protein reactivity within sera was assessed by either IgM or IgG binding, respectively, in ELISA. In **a**, ELISA index for PGL-I, LID-1, ML0405, and ML2331 of 107 leprosy patients' sera before treatment classified according to clinical forms and 200 sera from healthy household contacts (HHCs). The 107 patient sera were classified as: 17 tuberculoid (TT), 15 borderline tuberculoid-paucibacillary (BT-PB), 19 borderline tuberculoid-multibacillary (BT-MB), 19 borderline-borderline (BB), 14 borderline-lepromatous (BL), and 23 lepromatous leprosy (LL). A positive value was determined as an ELISA index> 1.1 when compared with responses of leprosy-endemic region control sera. #=p<0.001 versus HHCs. In **b**, the median ELISA index within each patient category immediately before and immediately after treatment is shown. *=p < 0.05 between pre- and post-treatment indices within the patient category

chimeric fusion protein that we recently described, LID-1 (comprising critical regions from ML0405 and ML2331), in sera from Venezuelan and Brazilian leprosy patients. As with anti-PGL-I responses, we found the highest levels of anti-LID-1 antibodies in LL patients, but absent or limited in TT patients. Thus, the IgG responses against each protein positively correlated with the bacillary load, clinical forms, and the operational classification at diagnosis, but alternative approaches appear to be required for the reliable diagnosis of PB patients. These results suggest that anti-protein antibody responses could be used to assist clinicians in determining the MDT regimen to provide patients.

The extended duration of treatment, as well as the skin discoloration caused by clofazimine, often prompts non-compliance during leprosy treatment [22]. A recent study conducted in the Philippines showed that the non-compliance rate with the WHO-provided MDT regimen

among study subjects can be as high as 30% in some leprosy-endemic regions [23]. Given the numerous reports of patients who retain significant numbers of *M. leprae* even upon completing a full recommended MDT regimen, non-compliance is a major concern for relapse. While most patients demonstrated negative results in ELISA years after treatment, it is noteworthy that three of the former Venezuelan patients who tested positive by antigen-specific antibody ELISA had previously received irregular MDT treatment for MB leprosy. These observations are consistent with a previous report documenting the retention of anti-PGL-I antibodies in a non-compliant patient [24]. The other former patient that tested positive by anti-LID-1 ELISA had been characterized as an indeterminate case and had been provided the shorter course of MDT intended for PB leprosy. Regular measurement of antibody levels throughout and even after treatment may identify those patients in need of further treatment.

It is well established that the earlier a leprosy patient is identified, the better their response to treatment. It stands to reason that the earlier ineffective treatment can be identified, the earlier an adjustment can be made to render treatment effective to improve outcome. Previous examination of anti-PGL-I responses have demonstrated reduced anti-PGL-I responses after treatment, with an approximate drop of approximately 50–90% in 2 years after the initiation of treatment [16–18, 25]. Our data suggest that protein-specific IgG antibodies decline more rapidly than anti-PGL-I IgM antibodies in leprosy patients under MDT. Protein-specific IgG antibodies were significantly reduced as early as three months after initial treatment, in contrast with the anti-PGL-I responses. Our observation that anti-PGL-I responses are not affected after the initial treatment is in agreement with a previous study [26]. The reasons for this disparity are unclear, but one suggestion would be that protein is cleared more rapidly from the infection site than glycolipid, removing an antigen reservoir that could perpetuate antibody production. The examination of former patients provided effective treatment indicated that the antibody responses are diminished for an extended period of time, such that the inclusion of former patients would not interfere with screening programs.

Interestingly, our data also support the measurement of IgG and IgM responses as prognostic markers for the re-emergence of the disease and suggest that patients should be discharged based on their immunological behavior during and after treatment. Persistent seropositivity appears to indicate a higher risk of developing recurrence of disease in the near future. Positive results would be indicative of sub-clinical infection, relapse, or re-infection, but not residual responses persisting from the initial *M. leprae* infection.

While WHO-provided MDT has had a large impact on leprosy case numbers; a recent report demonstrated that

Table 1 Proportion of positive antigen-specific antibody responses before and immediately after treatment (Uberlândia, Brazil)

	PGL-I		LID-1		ML0405		ML2331	
	Pre	Post[a]	Pre	Post	Pre	Post	Pre	Post
LL[b]	100	94	87	91	87	91	87	83
BL	100	80	100	79	100	79	79	50
BB	93	73	74	37	74	26	89	79
BT (MB)	83	50	53	53	53	26	32	37
BT (PB)	38	23	33	33	27	20	20	7
TT	14	14	20	13	13	7	33	7

[a] The duration of treatment was 6 months for PB and 12 months for most MB forms, with the exception of a 24-month treatment for LL

[b] n=23 for LL, 14 for BL, 19 for BB, 19 for BT (MB), 15 for BT (PB), and 17 for TT
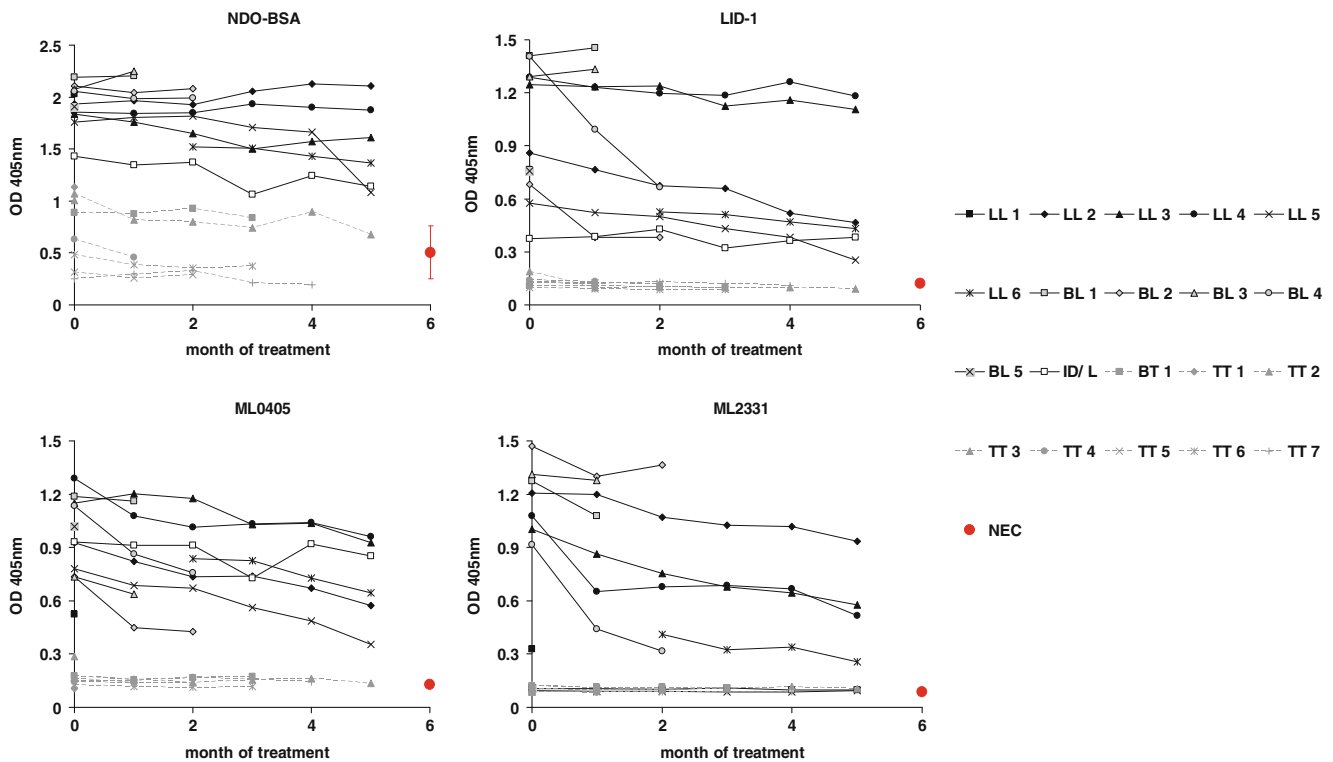
Fig. 4 Slow decline of antibody responses during MDT. NDO-BSA and recombinant protein reactivity within sera from a prospective study conducted in São Paulo, Brazil, was assessed by either IgM or IgG binding, respectively, in ELISA. Sera were collected at monthly intervals after the initiation of MDT and the results are shown as the optical density (OD) for each sample at each collection. The data point at month 6 designates the mean reactivity of non-endemic control (NEC) sera, along with the standard deviation (SD)

approximately 1 in 5 *M. leprae* isolates from biopsied patient samples were resistant to dapsone, rifampin, or clofazimine, and 1 in 16 were resistant to more than one drug [27]. Multidrug-resistant strains of *M. leprae* have been reported by several other investigators [9, 28–30], and conditions are often conducive for the further emergence of resistance [31]. The continued success of the current drugs, therefore, appears limited. While ofloxacin and minocycline have been added to the drug arsenal available for the treatment of leprosy, new anti-leprosy drugs are severely limited [32–35]. Without the development of improved therapies, the elimination of leprosy is unlikely. Studies examining new interventions or treatments for leprosy are hindered by the length of time required to reach clinical endpoints with which to determine success. Our data indicate that regular assessment of the anti-protein responses could provide intermediate readouts to aid in the more rapid assessment of new control strategies.

Our results suggest that the combination of LID-1 and PGL-I antigens, recognizing the IgG and IgM response, respectively, could be employed as an auxiliary tool in current control programs for leprosy diagnosis and treatment monitoring. Our data also demonstrate that the anti-protein IgG responses can be used as simple and objective

measures of leprosy treatment efficacy and as prognostic markers of relapse. Additionally, these biomarkers may also be employed as tools within trials of new treatments. In conjunction with our program aimed at developing rapid, point-of-care leprosy diagnostic tests, the identification of novel assessments of treatment efficacy could significantly impact patient care, provide improved outcomes, and sustain or improve the current level of leprosy control attained by the WHO-provided MDT.

Table 2 Rate of decline of antigen-specific antibody responses of LL patients during treatment. Antigen-specific responses were assessed during multidrug therapy (MDT) and compared against the response obtained at the time of diagnosis. Five LL patients, recruited in São Paulo, Brazil, were examined. The data are shown as a mean percentage versus initial response at diagnosis, with the range of responses indicated in parentheses. *=$p < 0.05$ and # = $p < 0.01$ versus time of diagnosis of the samples

Month of MDT	2	3	5
NDO-BSA	99 (90–104)	98 (82–106)	90 (62–109)
LID-1	91 (78–99)	86 (75–97)*	72 (44–92)*
ML0405	89 (79–103)	84 (72–99)*	68 (45–81)#
ML2331	85 (63–98)*	77 (63–92)#	69 (48–99)*

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