Rational Design and Evaluation of a Multipitope Chimeric Fusion Protein with the Potential for Leprosy Diagnosis

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Received 5 October 2009/Returned for modification 25 October 2009/Accepted 10 December 2009

Despite the reduction in the number of leprosy cases registered worldwide as a result of the widespread use of multdrug therapy, the number of new cases detected each year remains stable in many countries. This indicates that Mycobacterium leprae, the causative agent of leprosy, is still being transmitted and that, without an earlier diagnosis, transmission will continue and infection will remain a health problem. The current means of diagnosis of leprosy is based on the appearance of clinical symptoms, which in many cases occur after significant and irreversible nerve damage has occurred. Our recent work identified several recombinant antigens that are specifically recognized by leprosy patients. The goal of the present study was to produce and validate the reactivity of a chimeric fusion protein that possesses the antibody binding properties of several of these proteins. The availability of such a chimeric fusion protein will simplify future test development and reduce production costs. We first identified the antibody binding regions within our top five antigen candidates by performing enzyme-linked immunosorbent assays with overlapping peptides representing the amino acid sequences of each protein. Having identified these regions, we generated a fusion construct of these components (protein advances diagnostic of leprosy [PADL]) and demonstrated that the PADL protein retains the antibody reactivity of the component antigens. PADL was able to complement a protein that we previously produced (the leprosy IDRI [Infectious Disease Research Institute] diagnostic 1 [LID-1] protein) to permit the improved diagnosis of multibacillary leprosy and that had a good ability to discriminate patients with multibacillary leprosy from control individuals. A serological diagnostic test consisting of these antigens could be applied within leprosy control programs to reduce transmission and to limit the appearance of leprosy-associated disabilities and stigmatizing deformities by directing treatment.

Leprosy is typically classified on the basis of clinical manifestations and skin smear results. Simple guidelines have been suggested by the World Health Organization (WHO), with diagnostic criteria listed as being one or more of the following: hypopigmented or reddish skin patches with a definite loss of sensation, thickened peripheral nerves, and the appearance of acid-fast bacilli on analysis of skin smears and biopsy specimens (25a). In the classification based on skin smears, patients showing negative smears with samples from all sites are grouped as having paucibacillary (PB) leprosy, while those showing positive smears with samples from any site are grouped as having multibacillary (MB) leprosy. In practice, however, because skin-smear services are absent or unreliable, most programs use clinical criteria to classify individual patients and select their treatment regimens. The clinical system uses the number of skin lesions and the number of nerves involved to group leprosy patients into one of two simplified categories: MB leprosy (five or more lesions) and PB leprosy (less than five lesions). Thus, TT patients and most BT patients are categorized as having MB leprosy, while LL, BL, BB, and some BT patients are categorized as having PB leprosy.
otics in the form of multidrug therapy (MDT). For MB leprosy, a combination therapy employing rifampin, dapsone, and clofazimine is recommended for 12 months, while for PB leprosy a regimen with only rifampin and dapsone given over 6 months is recommended. It is particularly important to ensure that patients with MB disease are not undertreated with the regimen for the PB form of the disease. Thus, determination of an appropriate treatment regimen requires the accurate and differential diagnosis of the MB and PB forms.

A rapid, easy-to-use test that would simplify leprosy diagnosis could greatly assist with the prompt initiation of treatment. Tests based on IgM recognition of phenolic glycolipid I (PGL-I) have been used in a confirmatory role in the clinic (3, 4, 6, 14, 19, 20, 26). Testing for the presence of PGL-I-specific IgM gives a fairly high false-positive rate (>10%) in areas where leprosy is endemic, and while positive responses are indicated to be risk factors in the development of disease, the fact that many people with antibodies against PGL-I do not develop leprosy has hindered the widespread adoption of these tests in screening programs (4, 5, 14). For these reasons, additional antigens have been produced by our group and others with the goal of providing a clear, accurate, and rapid means of diagnosis of leprosy (8, 9, 15, 23).

In the study described here, we have extended our previous observations by creating a new polyepitope chimeric fusion protein with the potential to bind to serum antibodies of leprosy patients to provide a leprosy diagnosis. We refined our previous observations by determining antibody-reactive regions within select antigens in order to produce a synthetic protein that combines these reactive portions within a single product. Our results indicate that all portions contained within the synthetic protein retain their antibody binding activity and that this protein has utility for leprosy diagnosis.

MATERIALS AND METHODS

Subject and samples. Sera were obtained from patients with leprosy (10 patients with MB leprosy and 9 patients with PB leprosy in Sao Paulo, Brazil; and 20 patients with MB leprosy and 15 patients with PB leprosy in Cebu City, Philippines); from 10 controls in Cebu City, an area where leprosy is endemic (ECs); and from 8 U.S.-based control individuals, that is, individuals from an area where leprosy is not endemic (NECs). The sera from patients with MB and PB leprosy used in this study were derived from newly diagnosed, previously untreated individuals who did not have signs of reversal reactions. Sera were collected from 9 female and 10 male leprosy patients (age range, 22 to 63 years; average age, 30.9 years) recruited in Cebu City between 2007 and 2009. Leprosy was classified in each case by clinical and histological observations by creating a new polyepitope chimeric fusion construct that could greatly assist with the prompt initiation of treatment. For the natural disaccharide octyl (NDO)-BSA ELISA, PolySorp proteins or anti-human IgM for NDO-BSA (Rockland Immunochemicals, Gilbertsville, PA) diluted in 0.1% BSA-BPS was added to each well, and the plates were incubated at room temperature for 2 h with shaking. After the plates were washed, the plates were developed with tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and the reaction was quenched by the addition of 1 N H2SO4. The optical density of each well was read at 450 nm. Pools of sera from patients recruited in Sao Paulo were used for antibody optimization, and serum samples from individual Brazilian and Filipino recruits were used to evaluate the sensitivity and the specificity of the ELISA.

Target antigens. Antigens were produced as described previously (17). The reactive peptides from the initial peptide screen were reverse translated to design the PADL, and a codon-optimized gene for Escherichia coli was ordered from BlueHeron Biotechnology (Bothell, WA). DNA encoding the PADL was ligated into the pET29 vector and transformed into E. coli XL-10 competent cells. The PCR product was selectively removed by restriction enzyme digestion and directionally cloned into the expression vector pET29a (Novagen, Madison, WI) with a six-histidine tag incorporated at the N terminus. The sequence-verified expression construct was then transformed into E. coli HMS-174 to produce recombinant protein. The recombinant protein was isolated from washed inclusion bodies, resublimated in 8 M urea, and purified by anion-exchange chromatography (18) on a Q Sepharose Fast Flow (QFF) resin. The final product was affinity purified by the bichinonic acid protein assay (Pierce), the quality of the product was assessed by SDS-PAGE, and endotoxin contamination (<100 EU/mg protein for all) was measured by the Limulus amebocyte lysate QCL-1000 assay (Lonza Inc., Basel, Switzerland).

Generation and analysis of hyperimmune mouse serum. Six- to 8-week-old C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA), maintained under specific-pathogen-free conditions in the animal facilities of the IDRI. Recombinant proteins were formulated to provide final concentrations of 100 μg protein/ml and 200 μg EM005 adjuvant/ml (IDRI). Mice were immunized three times by the subcutaneous injection of 0.1 ml vaccine at the base of the tail at 2-week intervals. The animal procedures were approved by the IDRI institutional animal care and use committee. Mouse serum was collected at various times after the final immunization and was analyzed by antibody-capture ELISA. ELISA plates (Nunc) were coated with recombinant antigen in 0.1 M bicarbonate buffer and blocked with 1% BSA-BPS. Then, in consecutive order and following washes in PBS-Tween, mouse serum, anti-mouse IgG-horseradish peroxidase (Southern Biotech, Birmingham, AL), and 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) diammonium salt-H2O2 (Kirkegaard & Perry Laboratories) were added to the plates. The plates were read at 405 nm.

Statistics. The P values were determined by Student’s t test.

RESULTS

Determining reactive epitopes within selected M. leprae proteins. Our previous data, generated by protein array analysis, identified several proteins that are reactive with leprosy patient sera (9). In this investigation, we delineated to overlapping peptides those proteins that were useful for leprosy diagnosis (ML0405, ML2331, ML2055, ML0411, and ML0091) with the goal of identifying specific regions within each protein that can be used to refine and improve the ability to make a diagnosis. We included the ML0049 protein (an ESAT-6 homolog) and the ML0050 protein (a CFP-10 homolog) as controls within our peptide analyses. Peptides were synthesized at a size of 15 amino acids each and with a 5-amino-acid overlap to provide broad coverage across the amino acid sequences of each protein. The peptides were then reacted with leprosy patient sera in an ELISA. As expected, reactivity with peptides from each protein was identified (Fig. 1). By aligning the patterns of peptide reactivity from several patients, we were able to dis-
cern regions within each protein that yielded the greatest responses (Fig. 2A). These regions represent the antibody binding epitopes within each protein that can best be utilized for diagnosis.

**Generation and reactivity of synthetic protein.** To generate in a cost-effective manner a single product representing each of these epitopes whose quality could be controlled, we designed a polyepitope chimeric fusion protein that would display the two most reactive epitopes from each component antigen following expression as a recombinant antigen. This synthetic protein, designated PADL, was readily expressed and purified (Fig. 2B). As a preliminary screen of reactivity with patient sera and to optimize assay conditions, various concentrations of PADL were incubated in ELISA plates and reacted with two distinct pools of serum from Brazilian leprosy patients. We found good antibody reactivity within the serum pools tested and found that we required an ELISA coating concentration of at least 2.5 μg/ml PADL to saturate each well and generate the maximum attainable signal (Fig. 2C). These pools of leprosy patient serum were also reactive with the individual components of the PADL (data not shown), so we could not determine if the observed reactivity was due to all components, several components, or only a single component of PADL.

To demonstrate that each component of PADL could be recognized and contribute to the signal, anti-PADL serum was generated by hyperimmunizing mice, and this serum was used in a screen of reactivity of our synthetic protein and each component. The anti-PADL serum, but not normal mouse serum (NMS), displayed reactivity not only with the synthetic PADL protein but also with each protein from which sequences were selected (Fig. 3A). Similarly, the reacting antisera generated by hyperimmunizing mice with each individual protein represented in PADL with the PADL protein demonstrated that each epitope retained its antibody binding ability (Fig. 3B). These data indicate that PADL retains the ability to display each region in a manner suitable for antibody recognition and thereby effectively represents each of the five proteins from which regions were selected.

**PADL reactivity with patient sera.** To formally evaluate if PADL could be used for the diagnosis of leprosy, we performed PADL-specific ELISA with sera from patients with clinically diagnosed leprosy. Sera from well-characterized Brazilian leprosy patients were examined, and the anti-PADL responses were compared with the responses against the previously described diagnostic antigens, LID-1 and NDO-BSA (8, 9). The responses of the MB leprosy patients to NDO-BSA were strong, but strong responses were also observed for several serum samples from NECs (Fig. 4A). In general, the PADL responses mirrored the LID-1 responses, although sera from three MB leprosy patients gave a stronger signal for PADL than for LID-1. The opposite was also true, however, with sera from seven MB leprosy patients, which produced a
stronger signal for LID-1 than for PADL. For both recombinant antigens (LID-1 and PADL), the responses were not observed with any of the NEC sera. The results generated with sera from Brazilian leprosy patients were verified by evaluating the anti-PADL and the anti-LID-1 responses in the sera from Filipino leprosy patients and ECs. In these sera, the LID-1 responses were generally higher than the PADL responses, with LID-1 being detected by sera from 17 of 20 patients with MB leprosy but by none of the sera from patients with PB leprosy at a threshold of threefold above the average response of the ECs (Fig. 4B). None of the EC sera generated a LID-1 signal greater than this threshold. Even though PADL provided a weaker ELISA signal than LID-1, when they were tested against PADL, sera from 16 of 20 patients with MB leprosy and from 1 of the 15 patients with PB leprosy gave a positive response. LID-1 provided a strong signal with sera from most patients with MB leprosy, but the PADL responses for four of the serum samples were higher than the LID-1 signal. Interestingly, three of the four highest anti-PADL responses were detected in sera with LID-1 responses toward the lower end of the responses. Together, these results suggest the utility of both LID-1 and PADL for the clear diagnosis of MB leprosy.

**DISCUSSION**

Leprosy is believed to take up to 7 years to become manifest, but at present the diagnosis of leprosy is based on the emerg-
gence of clinical signs. Since sensory loss is one of the clinical signs used to identify leprosy, the earlier detection of *M. leprae* infection would be preferable and should be possible. We recently conducted screening with leprosy patient sera to identify novel *M. leprae* antigens that have not previously been described or evaluated to determine the diagnostic potential of these antigens (9). In this study, we refined our previous observations by use of overlapping peptides from the leading diagnostic candidate proteins to determine antibody-reactive regions. We then created and expressed a multiepitope chimeric fusion protein that combines these reactive portions within a single product (PADL). Leprosy patient sera typically reacted with more than one of the individual components of PADL to various extents, so we could not determine if the observed reactivity was due to all components, several components, or only a single component of PADL. The PADL ELISA with sera from mice immunized either with PADL or with each protein from which the components for PADL were selected indicated that each component of the synthetic protein retained antibody binding activity. Finally, antibodies in sera from MB leprosy patients but not control sera bound to PADL, demonstrating that this protein has utility for leprosy diagnosis.

Our results further demonstrate the utility of several *M. leprae* proteins for diagnosing leprosy. By delineating the antibody responses against proteins to responses against peptides, we limit our analyses to linear rather than conformational epitopes. It should be noted, however, that by expressing recombinant proteins in *E. coli*, even full-length proteins are probably not truly representative of the native *M. leprae* proteins, which lack the glycosylation states that mycobacteria create on their proteins. Thus, unless native proteins or proteins expressed in other mycobacteria are used, antibody-based tests will not be fully exhaustive and will likely exclude conformational epitopes that can be recognized during infection. Our data show that the majority of serum samples from MB leprosy patients can bind to *E. coli*-expressed synthetic proteins and indicate that limiting the responses to the detection of linear or nonnative epitopes is not prohibitive for antibody-based diagnosis.

The main principles of leprosy control are, at present, the timely detection of new cases and the prompt treatment of patients with MDT (25). By providing MDT free of charge, WHO has made significant progress in decreasing the global prevalence of leprosy. Unfortunately, although integration into other aspects of the health care system has occurred in many countries, this success has led to a corresponding erosion of leprosy clinics and specialists. As the transmission of *M. leprae* still appears to be occurring at a relatively consistent rate in many countries, the need for simple and practical tools for diagnosing leprosy in non-specialized settings would appear to be of the utmost importance.

In most patients, leprosy is diagnosed by passive case detection when they present to clinics. A recent large-scale active case-finding study involving the clinical examination of 17,862 residents in northwest Bangladesh indicates that the true prevalence rates in the region may be sixfold higher than those being determined by the use of traditional methods (12). Those findings are consistent with the findings presented in previous reports, in which active case finding returned prevalence rates much higher than those being reported (1, 11, 21). The development of simple tools, such as rapid lateral-flow-type tests containing either or both of the LID-1 and PADL proteins, could dramatically affect leprosy control programs by facilitating screening programs.

It is well documented that the household contacts of patients with MB leprosy have a higher risk of developing clinical leprosy than the contacts of patients with PB leprosy (7, 10). This increased risk has been attributed to the shedding and spread of viable bacteria by MB leprosy patients (2, 16). The earlier detection of leprosy cases would affect transmission by permitting the treatment of infected individuals before they inadvertently spread disease to others. In addition, it is well established that the earlier that a patient is identified, the better the

**FIG. 4.** PADL reacts with sera from MB leprosy patients. Antibody reactivity against NDO-BSA, LID-1, or PADL was assessed. (A) Sera from 19 patients with clinically diagnosed leprosy (10 with MB leprosy and 9 with PB leprosy) from Sao Paulo, Brazil, and 8 NEC individuals were examined. (B) Sera from 20 patients with MB leprosy, 15 patients with PB leprosy, and 10 ECs from Cebu City, Philippines, were examined. NDO-BSA reactivity was assessed by IgM binding, and recombinant protein reactivity was assessed by IgG binding. Sera were ranked by reactivity against NDO-BSA (A) and LID-1 (B).
individual's response to treatment is and the better the chance that the individual will avoid severe long-term nerve damage (13, 24). Thus, the accurate and early detection of M. leprae-infected individuals will open the possibility of earlier treatment that could both prevent disability and significantly affect leprosy transmission. Our results demonstrate that PADL can provide an accurate diagnosis of MB leprosy. Further research involving the collection and analysis of serum obtained in screening studies during which individuals may develop clinically confirmed leprosy are required to determine if PADL can detect M. leprae infection early in its development.

A test that could be used to screen the contacts of patients known to have leprosy on a regular basis or that could be applied with relative ease in regions where leprosy is hyperendemic might have a dramatic and long-lasting impact on new case detection rates. We are currently evaluating additional antigens, diagnostic formats, and patient sera from different geographic sources with the objective of attaining the early and simple diagnosis of leprosy regardless of the geographic location or experience of the examiner.

ACKNOWLEDGMENTS

This work was conducted with support from the National Institutes of Health (grants 1R43AI066613-01A1 and 2R44AI066613-02), American Leprosy Missions, and The New York Community Trust (Heiser Foundation). IDRI is a member of the IDEAL (Initiative for Diagnostics and Epidemiological Assays for Leprosy) Consortium.

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