

## Insight toward Early Diagnosis of Leprosy through Analysis of the Developing Antibody Responses of *Mycobacterium leprae*-Infected Armadillos<sup>∇</sup>

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**Leprosy is a debilitating chronic disease caused by infection with *Mycobacterium leprae*. A World Health Organization-directed control strategy based upon the identification and treatment of patients has resulted in a marked reduction in the number of registered worldwide leprosy cases over the last 20 years. Despite these efforts, the number of new leprosy cases detected each year now remains relatively stable, and *M. leprae* infection continues to pose a health problem. It is suggested that earlier diagnosis is required to strengthen control programs. In this study, we have examined the development of antigen-specific immunoglobulin responses within armadillos experimentally infected with *M. leprae* to identify those responses that develop most rapidly and robustly following infection. Antibody responses to the *M. leprae*-specific phenolic glycolipid I and several protein antigens previously demonstrated to have diagnostic potential were assessed. Our results identify several antigens that can provide early diagnosis of *M. leprae* infection but also indicate considerable variability in the development of antigen-specific antibodies. Our data suggest that a combination of antigens is likely required to provide accurate and early leprosy diagnosis.**

Leprosy is caused by infection with the bacterium *Mycobacterium leprae*, and its clinical symptoms, bacterial burdens, pathology, and underlying immunological responses vary widely. Diagnosis is complicated due to this wide range of signs but can be arranged histologically into the five distinct categories of the Ridley-Jopling scale (polar tuberculoid [TT], borderline tuberculoid [BT], mid-borderline [BB], borderline lepromatous [BL], and polar lepromatous [LL]) (22, 24). The World Health Organization (WHO) has suggested diagnosis of leprosy be made by the observation of one or more of the following: hypopigmented or reddish skin patches with definite loss of sensation; thickened peripheral nerves; and acid-fast bacilli on skin smears/biopsy specimens. In the classification based on skin smears, patients showing negative smears at all sites are grouped as paucibacillary (PB) leprosy, while those showing positive smears at any site are grouped as multibacillary (MB) leprosy. In practice, most programs use the clinical criteria for classifying and selecting the treatment regimen for individual patients. The clinical system includes counting the number of skin lesions and nerves involved as the basis for grouping leprosy patients into the PB (less than 5 lesions) and MB (typically 5 or more lesions) categories. It would be highly beneficial to identify *M. leprae* infection before such signs appear.

It is believed that a significant number of individuals contain infection such that it remains subclinical or self-cures with

minimal sign of disease (2, 11). If left untreated, however, leprosy can progress to a state of high significance and result in irreversible nerve damage with profound sensory and motor nerve loss, deformity, and blindness. It is well established that the earlier a leprosy patient is diagnosed and treated, the better their outcome (12, 19). It stands to reason that identifying leprosy patients on the basis of antigen-specific responses, preferably before the onset of symptoms, could have a dramatic effect on clinical outcome.

A major obstacle in research to develop new diagnostic tests for leprosy has been the inability to cultivate *M. leprae* *in vitro*, rendering it difficult to produce sufficient quantities of highly purified, defined research reagents. This hurdle has been lowered by the recent description of the *M. leprae* genome, which has made it easier to select and express recombinant antigens of potential diagnostic value (5). Another hurdle has been the difficulty in constructing field studies to capture samples from individuals who are in the early stages of leprosy. Studies conducted in the Philippines and Brazil, focusing on sample collection from at-risk populations, required the enrolment of large numbers of individuals with decades of follow-up to serendipitously recruit people who developed leprosy after the initial screening (6, 7, 14). Sample collection was generally conducted at extended intervals, limiting the ability to finely discriminate which responses develop the earliest. A final obstacle has been the paucity of a small-animal model of disease progression, rendering it difficult to mimic leprosy development. Immune-competent mice control bacterial burdens to various extents, do not develop nerve damage, and have relatively poor immune responses that preclude their use for diagnostic investigations (24). Immunocompromised mice do not

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TABLE 1. Characteristics of experimentally infected armadillos

Armadillo	Identifier	<i>M. leprae</i> burden at end of expt		Protein(s) that elicited earliest response(s) <sup>a</sup>	Earliest time of response (mo)	Protein(s) that elicited largest response(s) <sup>b</sup>
		Liver (10 <sup>9</sup> /g)	Spleen (10 <sup>10</sup> /g)			
1	8A25	2.43	1.60	NDO-BSA, <sup>c</sup> ML2346	14, 29	ML2346
2	1X	1.15	6.60	ML0411	9	PADL
3	2X	0.51	0.47	NDO-BSA <sup>c,d</sup>	12	NDO-BSA <sup>d</sup>
4	6R36	1.04	0.59	NDO-BSA <sup>c,d</sup>	5	PADL
5	7M44	1.72	0.22	LID-1, NDO-BSA, <sup>c,d</sup> ML0411, PADL, ML2346	11	PADL
6	6I63	1.10	0.41	LID-1, NDO-BSA, <sup>c,d</sup> ML0411	11	LID-1, PADL
7	6H49	1.76	1.74	NDO-BSA <sup>c,d</sup>	3	PADL
8	6G23	1.79	6.26	LID-1, NDO-BSA <sup>c</sup>	6	LID-1, PADL
9	5B33	5.53	2.93	NDO-BSA <sup>c,d</sup>	3	PADL
10	8A80	0.65	0.03	NDO-BSA <sup>d</sup>	2	PADL

<sup>a</sup> Responses against recombinant proteins or NDO-BSA detected with protein A were considered positive when a value greater than 3 times the preinoculation levels was detected. Anti-NDO-BSA responses detected with anti-IgM antibodies were considered positive when values of >700 units were attained.

<sup>b</sup> The largest response was determined as the highest OD value regardless of time.

<sup>c</sup> Detected with anti-IgM antibodies.

<sup>d</sup> Detected with protein A.

control bacterial replication but, as a consequence of their deficiencies, are not suitable for analyzing antibody responses.

Nine-banded armadillos (*Dasypus novemcinctus*) are natural hosts of *M. leprae*. The manifestation of leprosy in armadillos is strikingly similar to that in humans, presenting over a broad spectrum that is histopathologically classifiable from lepromatous to tuberculoid. Most significantly, armadillos also develop extensive nerve involvement with *M. leprae* and can exhibit many clinical conditions that are associated with the disease in humans, such as foot ulcers, skin lesions, and even blindness (17, 24, 26). In this study, we analyzed the developing antibody responses during experimental *M. leprae* infection of armadillos to identify antigens that may be used for early diagnosis of leprosy. Armadillo sera were collected before and after infection, and antigen-specific antibody responses determined. Our data identify several antigens that could be used in combination to provide early leprosy diagnosis.

#### MATERIALS AND METHODS

**Armadillos and experimental infection.** Nine-banded armadillos were trapped in the wild in western Arkansas and central Mississippi, areas known to be free of enzootic infection, and conditioned for captivity for *in vivo* propagation of *M. leprae* (29). All animals were adults in robust condition and weighed  $\geq 3.5$  kg prior to infection. Before study inclusion, each armadillo was classified according to its intradermal granulomatous response to  $1 \times 10^7$  heat-killed *M. leprae* prepared as lepromin A. Sites were biopsied and examined histopathologically after 21 days according to the procedure previously described (15). Animals classified as lepromatous were then infected by intravenous injection with  $1 \times 10^9$  bacilli prepared from serial passage of established cultivar strains in the footpads of nude mice (27). The animals were examined daily, and serum was obtained at least once every 3 months to monitor the immunological response to infection. The animals were routinely monitored for intercurrent issues and managed individually to obtain a maximum level of bacterial proliferation. At a time based on clinical observation (overall physiological decline and a specific algorithm, including phenolic glycolipid 1 enzyme-linked immunosorbent assay [PGL-I ELISA] IgM and circulating levels of the liver enzymes alanine aminotransferase, aspartate aminotransferase, and lactate dehydrogenase), usually 18 to 24 months after infection, each animal was humanely sacrificed and infection scored by direct microscopic count, indexing the level of bacterial proliferation in each gram of reticuloendothelial tissue, and was further classified in each case by histological observations carried out by qualified personnel (Table 1). The animal-handling protocol was conducted with all appropriate Office for Animal Welfare assured ethical safeguards (approval number A3032-01).

**Antibody responses of infected armadillos.** Armadillo sera were collected and stored at  $-70^\circ\text{C}$  until analysis by antibody capture ELISA against NDO-BSA (a synthetic derivative of PGL-I) and the previously described recombinant anti-

gens ML0091, ML0098, ML0276, ML0405, ML0411, ML0840, ML2028, ML2038, ML2055, ML2331, ML2346, LID-1 (a fusion of ML0405 and ML2331), and PADL (comprising epitopic regions from ML0405, ML2331, ML2055, ML0411, and ML0091) (7, 9). The properties of these proteins are described in Table 2. Polysorp plates (Nunc, Rochester, NY) were coated with 1 to 5  $\mu\text{g}/\text{ml}$  recombinant antigen in 0.1 M bicarbonate buffer and blocked with 1% bovine serum albumin (BSA)-phosphate-buffered saline (PBS). Then, in consecutive order and following extensive washes in PBS-Tween 20, armadillo sera (diluted 1/200) and protein A-horseradish peroxidase (Southern Biotech, Birmingham, AL) or goat anti-IgM antibodies (Cappell, Solon, OH) were added to the plates. Development was achieved by the addition of 2,2'-azino-bis(3-ethylbenzothiazolinesulfonic acid) (ABTS)-H<sub>2</sub>O<sub>2</sub> (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and stopped by the addition of 1 N sulfuric acid after approximately 15 min. Plates were analyzed at 450 nm (ELx808; Bio-Tek Instruments, Inc., Winooski, VT).

**Statistics.** Responses detected with protein A were considered positive when a value greater than 3 times the preinoculation level was detected. Responses detected with anti-IgM antibodies were considered positive when values of >700 units were attained.

#### RESULTS

**Detection of armadillo antibody responses.** Previous data from our group and others have indicated that IgM antibody responses against PGL-I (or its synthetic derivative NDO-BSA) and IgG responses against the chimeric fusion protein LID-1 (comprised of ML0405 and ML2331) can provide an early prognostic indicator of leprosy development (6, 7, 14). To confirm that armadillos also develop antibody responses to these antigens, we first examined whether sera from a pool of *M. leprae*-infected armadillos demonstrating a high level of anti-PGL-I antibodies reacted with these proteins. When ELISAs were conducted with recombinant protein and developed with either protein A or protein G, strong responses were detected in the infected but not in the negative-control serum (Fig. 1 and data not shown). Although rabbit anti-armadillo IgG yielded positive responses for infected armadillo sera against LID-1, significant reactivity with uninfected control armadillo sera indicated a lack of specificity, and the discrimination of positive and negative results was lower than that achieved with protein A (data not shown). For all subsequent recombinant-protein ELISAs, armadillo antibodies were detected by binding of protein A.

**Developing anti-NDO-BSA and anti-LID-1 responses in *M. leprae*-infected armadillos.** To determine the kinetics by which

TABLE 2. Characteristics of recombinant proteins assessed

Antigen	Size (aa) <sup>a</sup>	<i>M. tuberculosis</i> homolog (% identity)	Function and notes
ML0091	236	Rv3810 (52.7)	Exported repetitive protein precursor PirG; 28-kDa antigen precursor, cell surface protein
ML0098	301	Rv3803c (83.3)	Ag85C; <i>fbpC</i>
ML0276	147	Rv0390 (79.0)	Conserved hypothetical protein
ML0405	394	Rv3616c (62.7)	Conserved hypothetical protein
ML0411	446	Rv3883c (34.4)	Possible secreted protease
ML0840	434	None	Unknown
ML2028	327	Rv1886c (83.3)	Ag85B
ML2038	159	Rv1876 (86.0)	MMP-II; bacterioferritin
ML2055	287	Rv1860 (66.8)	ModD; fibronectin attachment protein
ML2331	256	Rv3717 (82.0)	Possible secreted protein
ML2346	301	None	Conserved hypothetical protein
LID-1			Chimeric fusion of ML0405 and ML2331
PADL			Chimeric fusion of B cell epitopic regions from ML0405, ML2331, ML2055, ML0411, and ML0091

<sup>a</sup> aa, amino acids.

armadillo antibody responses develop subsequent to *M. leprae* infection, sera were collected from armadillos at various intervals before and following experimental infection. We first examined the responses to NDO-BSA and LID-1. Similar patterns were observed for anti-NDO-BSA responses detected by either anti-IgM antibodies or protein A, with the exception of armadillos 1 and 8, in which protein A failed to detect the response (Fig. 2). Surprisingly, however, not all of the armadillos recognized LID-1 and not all of the armadillos recognized NDO-BSA. Rather, three armadillos (numbers 2, 3, and 9) had antibody responses against NDO-BSA that increased over time but these armadillos did not develop antibody responses against LID-1. In contrast, armadillo 8 had a strong antibody response against LID-1 but no protein A response against NDO-BSA, and armadillo 10 had an intermediate response to LID-1 but weak responses to NDO-BSA. With the exception of armadillo 1, which had only a weak positive response against NDO-BSA detected by anti-IgM antibodies (848 units after 14 months), the remaining armadillos developed strong responses to either the LID-1 or NDO-BSA antigens.

We have previously published data showing that PGL-I antibody levels in armadillos correspond to the level of bacterial dissemination in the tissues (28). The antibody responses to

the proteins tested here generally demonstrate the same pattern, although we did not observe any association between the profile of the response and the *M. leprae* burden determined at the end of the experiment (Table 1). For example, armadillo 9 had the highest liver burden but did not produce an anti-LID-1 response, while armadillo 8 had the third-highest liver burden but did not produce an NDO-BSA/protein A response. In general, the antibody responses increased over time before appearing to plateau or even slightly decrease as the infection reached its terminal phase.

**Ability of additional proteins to distinguish *M. leprae* infection.** Having demonstrated the ability of a protein antigen (LID-1) to indicate progressive *M. leprae* infection in armadillos, we expanded our analyses to examine several other proteins that are recognized during the immune responses of leprosy patients (8, 9, 16, 21). We first assessed the ability of each antigen to distinguish sera from an armadillo infected with a high dose of *M. leprae* from sera obtained from an uninfected armadillo. Specific antibodies were observed against several antigens (ML0091, ML0405, ML0840, ML2028 [Ag85B], ML2038 [MMP-II], ML2055, ML2331, ML2346, and the multiepitope chimeric fusion protein PADL) (Fig. 3). These data indicate that these proteins could be used to examine the progress of *M. leprae* infection in armadillos.

When these proteins were evaluated against the sera obtained sequentially following *M. leprae* infection, we found that each protein that had bound the positive-control sera could provide diagnosis of infected armadillos at various times (Fig. 4). The ML0411 protein detected responses sooner than these additional proteins in 4 armadillos (armadillos 2, 5, 6, and 9), each within the first year of infection (Fig. 4), whereas longer times were required to detect responses against most of the other proteins. Of these additional proteins, PADL and ML2346 provided the most robust responses, being strongly recognized by antibodies in 8 of 10 (80%) of the armadillos within 15 months of infection (Fig. 4). The other proteins tested could also detect varying levels of antibodies in the same animals (Fig. 4). ML2346 was the only protein evaluated that could identify *M. leprae* infection in armadillo 1, with anti-ML2346 responses detected in the penultimate and final serum

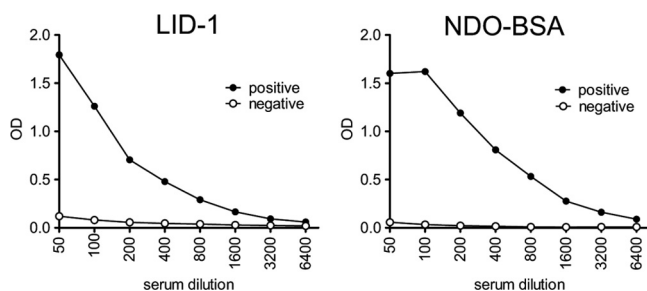


FIG. 1. Armadillo antibodies can be detected with protein A. Pooled sera from either uninfected control armadillos (negative) or armadillos heavily infected with *M. leprae* (positive) were assessed for reactivity against NDO-BSA and LID-1, using protein A as the detection reagent. Serum was diluted to determine the linear range of responsiveness. OD, optical density.

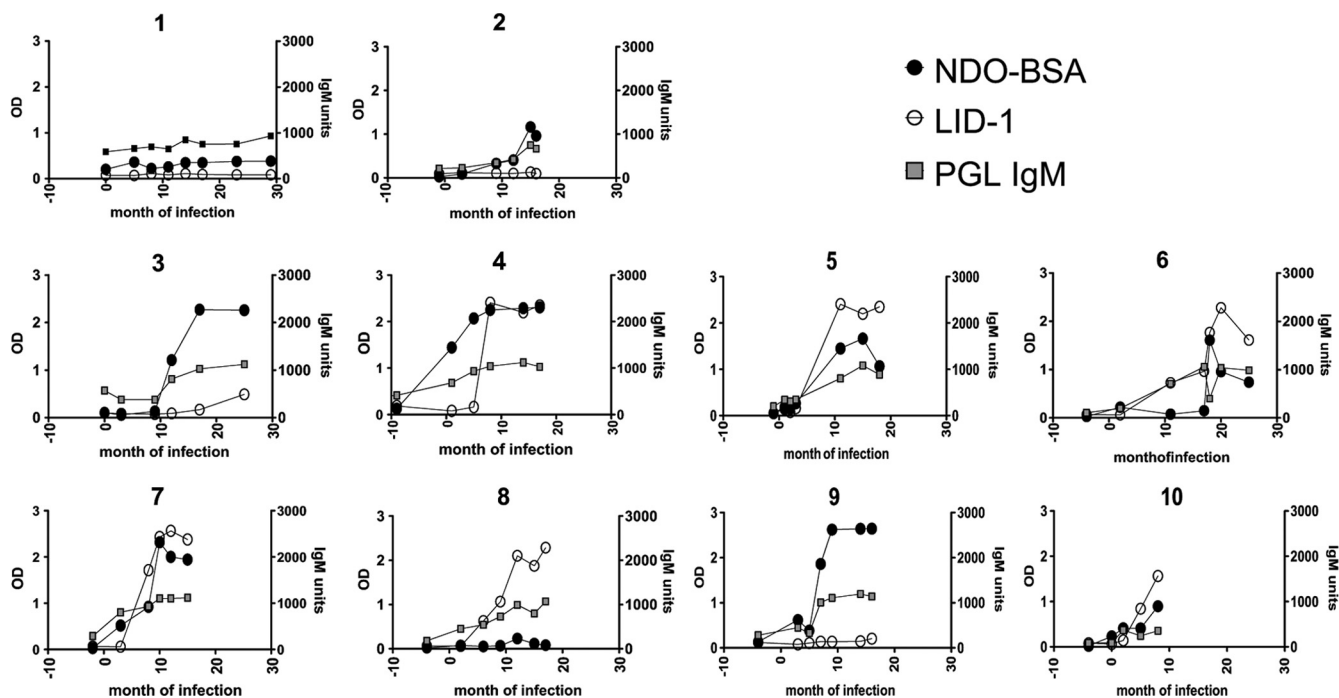


FIG. 2. Developing antibody responses of *M. leprae*-infected armadillos. Sera were collected sequentially from armadillos experimentally infected with *M. leprae*, and specific antibodies assessed against NDO-BSA and LID-1 by ELISA, using protein A (left y axis) or anti-IgM antibodies (right y axis) as the detection reagents. Each plot depicts the results for a single armadillo, with each point representing an individual time in relation to the inoculation of *M. leprae*. The data presented are results from one of two identical ELISAs performed on different days. OD, optical density.

sample collected (2 years after infection) (Fig. 4). In general, NDO-BSA and LID-1 provided the most rapid diagnosis of infected armadillos (Table 1). These data demonstrate that rising antibody responses are indicative of progressive *M. leprae* infection and strongly suggest that it is necessary to assess the antibody response against more than one antigen to provide thorough, accurate, and early diagnosis of leprosy.

DISCUSSION

More rapid diagnosis and administration of treatment are regarded as critical measures to ensure that current control strategies continue to reduce the burden of leprosy. Studies targeting early diagnosis of patients are notoriously inefficient and require a significant degree of expertise to ensure proper diagnosis. In this study, we used experimental *M. leprae* infection of nine-banded armadillos to provide a controlled time of infection against which to measure the development of antibody responses against a panel of potential diagnostic antigens. Our results indicate an inherent variability in the antigens that are recognized during infection and that these antigen-specific antibody responses can emerge at different times, but they indicate that a combination of the NDO-BSA and LID-1 antigens may be best suited to provide early and accurate leprosy diagnosis.

One approach for the early detection of *M. leprae* infection is through serological diagnosis, but very few studies have actually been undertaken with a defined goal of examining the development of leprosy-related antibody responses. Studies screening household contacts of MB leprosy patients, who are known to be at a higher risk of developing leprosy than the general population, have been conducted. Such studies represent a huge undertaking, requiring labor-intensive, clinical follow-up of large numbers of contacts over extended periods of time in order to obtain sufficient numbers of patients (6, 14). These screening exams identified a small percentage of individuals recruited to the studies who developed symptomatic

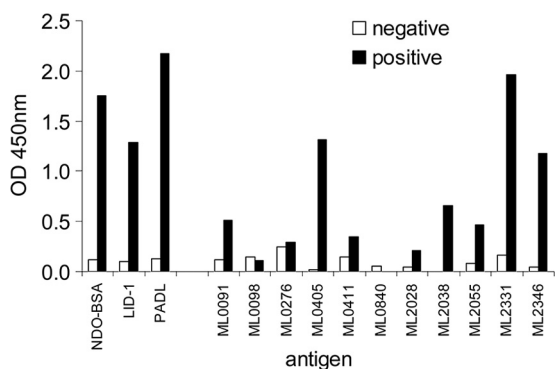


FIG. 3. Antigen-specific antibody responses of *M. leprae*-infected armadillos. Sera from uninfected (negative) controls or armadillos infected with a high dose of *M. leprae* (positive) were assessed for reactivity against various recombinant *M. leprae* proteins. Serum was diluted 1/200 for the assay. OD<sub>450</sub>, optical density at 450 nm.

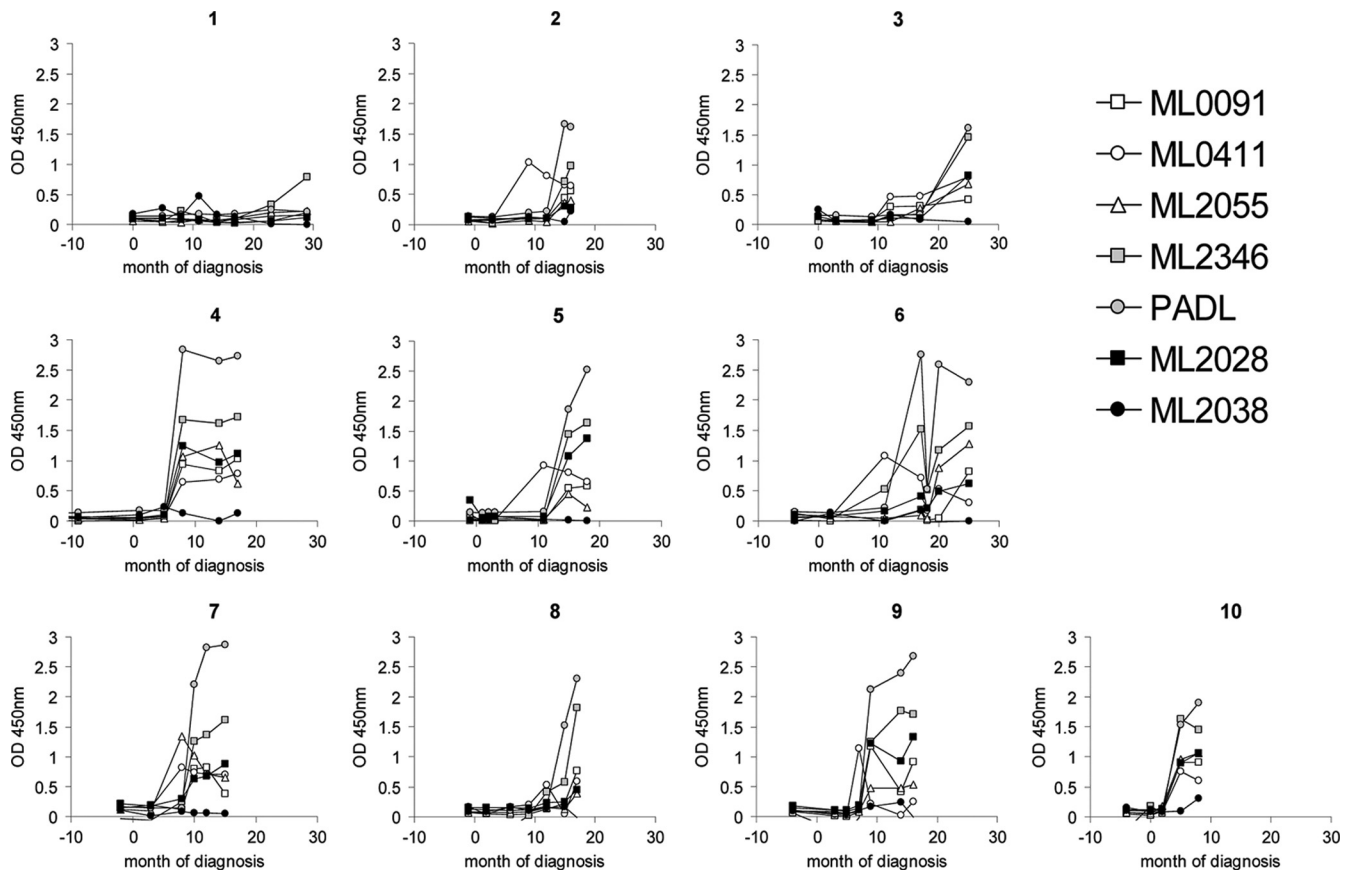


FIG. 4. Developing anti-protein antibody responses of *M. leprae*-infected armadillos. Sera collected sequentially from armadillos experimentally infected with *M. leprae* were assessed against antigens yielding positive results against control sera. Each plot depicts the response of an individual armadillo, with each line representing a single protein. OD<sub>450</sub>, optical density at 450 nm.

leprosy at some point during the follow-up, thereby serendipitously providing presymptomatic samples. An alternative strategy is to examine the development of serologic antibody responses during experimental *M. leprae* infection, while acknowledging the caveat that responses may differ between species. Armadillos develop pathology similar to that of leprosy patients, and we therefore reasoned that controlled experimental infection of armadillos could reveal the antigen-specific antibody responses that emerged earliest in the infectious process and thereby indicate those proteins most likely to provide early diagnosis in humans. Following intravenous inoculation of *M. leprae* in armadillos, the earliest sign of the infection appears some 6 months later as a sustained evolution of IgM antibodies to the PGL-I antigen, and the initiation of the PGL-I IgM response can be associated with the establishment of *M. leprae* to a 1+ bacterial index in some reticuloendothelial tissues, such as liver, spleen, or lymph node (28). Our results not only demonstrate that armadillos recognize several antigens that are also recognized by leprosy patients but identify discrepancies in the antigen-specific antibody responses that develop. In some armadillos, the response to NDO-BSA (PGL-I) developed more rapidly than the LID-1 response, but the opposite was also observed, with the LID-1 response developing more rapidly than the NDO-BSA response in other armadillos. This observation suggests that the best strategy for

identifying leprosy as early as possible may be to integrate both of these antigens into tests.

A simple serum antibody-based diagnosis could be readily adapted to rapid test formats that can be applied in field studies, negating the need for individuals to present at or be referred to clinics (10). The field adaptability of screening tests is critical for ensuring that large numbers of individuals can be evaluated without putting an undue burden on clinical staff. The composition of antigens to include in such a test is not yet clear for leprosy. Rapid tests based solely on PGL-I derivatives have been developed and distributed but are not currently in widespread use and are somewhat restricted by high false-positive rates within regions where leprosy is endemic and even in regions where it is not endemic (3, 4, 13, 20). Our data indicate that while NDO-BSA can provide early diagnosis in armadillos, this can also be achieved with several protein antigens. Our previous data indicated that many of these protein-specific antibody responses are restricted to leprosy patients and have a limited rate of false-positive results. Armadillos are exposed to a wide variety of mycobacteria in the environment, but our data indicate that such exposures generate neither long-term nor prime cross-reactive responses against the particular *M. leprae* antigens we analyzed. Such protein antigens could be used to both complement and supplement a PGL-I-based diagnosis. This not only might improve the accuracy of

the test but could also provide an earlier time during *M. leprae* infection at which rapid tests could diagnose leprosy.

As transmission of *M. leprae* is still occurring at relatively consistent rates and drug resistance is emerging, the need for simple and practical tools for diagnosing leprosy in nonspecialized clinics rises. Most leprosy patients are diagnosed by passive case detection when they present to clinics after symptoms have persisted for some time. A recent large-scale active case-finding study involving clinical examination of 17,862 residents in northwest Bangladesh indicates that the true prevalence rates in the region may be 6-fold higher than those being reported by traditional methods (18). Those findings are consistent with previous reports in which active case finding returned much higher prevalence rates than those being reported (1, 23). The identification of responses that provide an early indication of disease onset, such as the responses indicated during experimental infection of armadillos outlined in this report, could have a dramatic impact on leprosy on both an individual and a population basis.

Our results identify antigens best suited to provide early leprosy diagnosis in the experimental setting of armadillo infection. These data can inform screening programs within regions where leprosy is endemic with a goal of earlier identification of leprosy patients. Accurate and early detection of *M. leprae*-infected individuals will open the possibility of earlier treatment that could contribute both to prevention of disability and to significantly reducing leprosy transmission.

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