The Evolution of IgG and IgM Antibodies to Antigens of Mycobacterium Leprae in Experimentally Inoculated Armadillos.

Abdul Reza Vadiee

Louisiana State University and Agricultural & Mechanical College

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The evolution of IgG and IgM antibodies to antigens of *Mycobacterium leprae* in experimentally inoculated armadillos

Vadiee, Abdul Reza, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1989
THE EVOLUTION OF IgG AND IgM ANTIBODIES TO ANTIGENS OF MYCOBACTERIUM LEPRAE IN EXPERIMENTALLY INOCULATED ARMADILLOS

A dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Department of Microbiology

by

Abdul Reza Vadiee
B.S., Southeastern Louisiana University, 1983
M.S., Southeastern Louisiana University, 1985
December, 1989
DEDICATION

To my parents and my wife, Carol Ann for their continuous support and for believing in me.
ACKNOWLEDGEMENT

A very sincere thank you is extended to my major Professor, Dr. Edward J. Shannon, for his advice, patience, helpful direction, and mature guidance throughout the course of this research. I would also like to thank Dr. Thomas P. Gillis for familiarizing me with several techniques and for his interest and encouragement throughout much of this research.

I would like to thank Dr. Robert C. Hastings for providing the facilities in the Pharmacology and Immunology Research Department laboratories of the Gillis W. Long Hansen's Disease Center. Thanks also go to Mr. Melvyn J. Morales for his help in preparing the figures, Ms. Tanya Thomassie and Mrs. Cindy Henk for photography.

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ABSTRACT

The nine-banded armadillo (Dasypus novencinctus linn.) is highly susceptible to infection with Mycobacterium leprae and has become an important animal model in the study of leprosy. In an attempt to define the major immunogens of M. leprae and the isotypic antibody response to these molecules during infection, the IgG and IgM antibody responses of experimentally-infected armadillos to the sonicated M. leprae, supernatant fraction of sonicated M. leprae, lipoarabinomannan (LAM) derived from M. tuberculosis, and phenolic glycolipid-I was analyzed using immunoblot and ELISA. Antibodies to the specific epitope (defined by monoclonal antibody IIIE9) on the 65-kDa protein of M. leprae were analyzed using a competitive antibody binding assay. Our results showed that protein antigens of M. leprae elicited a predominant IgG antibody response, whereas, carbohydrate antigens of M. leprae, including lipoarabinomannan, induced an IgM response. Phenolic glycolipid-I elicited both IgG and IgM antibody responses. Some animals produced and maintained a strong IgG antibody response to phenolic glycolipid-I which correlated with their ability to delay dissemination of the infection and with their ability to survive infection for longer periods of time. Antibodies to an M. leprae specific epitope on the 65-kDa protein in crude cell wall extracts but not to the recombinant 65-kDa protein were detected during the later stage of infection in both armadillos and leprosy patients. The lack of concordance between CABA's developed with the crude native antigen and those developed with the purified recombinant 65-kDa protein is suggestive of
false-positive results, possibly induced by steric hindrance, being associated with CABA which incorporate crude cell wall extracts as antigen source. Therefore, the IIIE9 epitope of M. leprae does not appear to be immunogenic in the armadillo or the leprosy patient.

It was shown that antibody profiles to various M. leprae antigens proved useful in monitoring the course and outcome of infection in armadillos as well as establishing baseline information on the humoral immune response in armadillos during the course of an untreated infection with M. leprae.
INTRODUCTION

After the discovery by Armauer Hansen in 1873 that *Mycobacterium leprae* is the causative agent of leprosy, *M. lepra*e remains to be one of the few microorganisms yet to be cultivated in vitro (22).

Man has traditionally been thought to be the only natural host of *M. lepra*. However, studies beginning in 1968 demonstrated that the nine-banded armadillo (*Dasypus novemcinctus*, linn.) is susceptible to experimental infection with *M. lepra* (15).

Although the *Dasypus novemcinctus* has teeth, the armadillo is a mammal of the zoological order, Edenthata (toothless). Its suborder is Xenarthra and its family is Dasypodidae. Armadillos are primarily carnivorous, but they also consume vegetable matter. Armadillos have a life-span of up to 15 years. Their reproduction is unusual in that mating and fertilization of the egg takes place some time in July or August while implantation of the fertilized egg takes place in December (23). Armadillos have a relatively low body temperature of 32 - 35°C (1) (89.6 - 95.0°F) and it is thought that this characteristic allows the leprosy bacillus to disseminate into tissues like the spleen and liver.

Successful transmission of leprosy to nine-banded armadillos by Kirchheimer and Storrs (15) has provided the investigators with sufficient sources of *M. lepra*e for antigenic analysis and vaccine development. Although armadillos do not exhibit reational states such as erythema nodosum leprosum (ENL), a leprosy complication, upon their exposure to *M. lepra*, they may exhibit a clinical manifestation of leprosy ranging from numerous acid-fast bacilli
(AFB) (lepromatous leprosy) to few AFB (tuberculoid) (14). However, the lepromatous type of leprosy is most common among armadillos.

Armadillos have become of interest to leprosy investigators because the dissemination of the disease is similar to lepromatous human patients. In the immunology of leprosy, armadillos offer a major advantage in that its immune response can be analyzed in the absence of anti-leprosy chemotherapy, an issue which ethically would not be possible in human patients. Additionally, they may be studied in the absence of immunosuppressive drugs such as corticosteroids, which are frequently used for the management of reactional states in leprosy such as ENL or reversal reactions.

Published findings describing immunological responses of armadillos to *M. leprae* have been few and to date we do not have a concise understanding of their immune response. Using a radioimmunoassay, Harboe, et al. (10) demonstrated increased amounts of antibodies to *M. leprae* antigen 7 during the course of experimental infection. In immunoblotting studies using 125I-labeled protein A, Chakrabarty, et al. (3) have shown the presence of antibody to several antigenic components of *M. leprae* in the sera of infected armadillos. A deficiency in these studies is the lack of demonstration of isotypic (IgG and IgM) antibody response. Recently, Truman, et al. (24) have shown that longitudinal IgM antibodies of armadillos to an *M. leprae*-specific antigen, phenolic glycolipid-I (PGL-I), (11) can be useful in following the course of infection. These investigators did not describe the evolution of IgM antibodies to PGL-I in individual animals and as a function of time.
Due to the need for a systematic and detailed study regarding the immune response of armadillos, this study was initiated to: 1) describe and chemically characterize the antigens of M. leprae that evoke IgG and IgM antibodies in armadillos and 2) to analyze the antibody response of armadillos to the major immunogens of M. leprae, including the purified PGL-I and an M. leprae-specific epitope located on a 65-kDa protein (2), during the course of an experimental inoculation with M. leprae.
Chapter I  Partial characterization of antigens from *M. leprae* evoking IgG and IgM antibodies in armadillos.
Dear Dr. Hastings:

I am in the process of writing my dissertation which will partially fulfill the requirements for the degree of Doctor of Philosophy in the Department of Microbiology at Louisiana State University in Baton Rouge. With your permission, I would like to include a reprint of the article entitled:

"Partial Characterization of Antigens from M. leprae Evoking IgG and IgM Antibodies in Armadillos"

By Abdul R. Vadiee, et al.,

which appeared in:


The bound dissertation and a microfilmed copy will become part of the collection of the university library. Your attention to this matter is greatly appreciated. Thank you very much.

Sincerely,

Abdul R. Vadiee
Partial Characterization of Antigens from *M. leprae*
Evoking IgG and IgM Antibodies in Armadillos¹,²

Abdul R. Vadiee, Edward J. Shannon,
Thomas P. Gillis, and Robert C. Hastings³

The nine-banded armadillo (*Dasypus novemcinctus*) is highly susceptible to infection with *Mycobacterium leprae* and has become an important animal model in the study of leprosy (⁶). Results describing serologic responses to *M. leprae* in armadillos have implicated several distinct antigenic components of the bacillus as major immunogens. These include, antigen 7 (⁴), cell wall polysaccharides (¹), and the unique glycolipid molecule, phenolic glycolipid-1 (PGL-I) (⁴).

In an attempt to define other major immunogens and the isotypic antibody response to these molecules, we analyzed the IgG and IgM antibody responses of heavily infected armadillos to the supernatant fraction from sonicated *M. leprae* antigens including the purified lipoarabinomannan (LAM) derived from *M. tuberculosis*. Our results showed that the predominant IgG antibody response of heavily infected armadillos was to protein antigens of the bacillus. In contrast, IgM antibodies from heavily infected armadillos, as well as from uninfected armadillos, were reactive with a major carbohydrate antigen of *M. leprae*.

**MATERIALS AND METHODS**

**Fractionation of armadillo serum by Sephacryl S-200.** An IgG-enriched fraction of normal armadillo pooled sera was obtained by molecular sieve chromatography on a 90 x 1.5 cm Sephacryl S-200 superfine column (Pharmacia Fine Chemicals, Piscataway, New Jersey, U.S.A.). The column was equilibrated with 0.1 M Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl. Two ml fractions were collected at a flow rate of 12 ml per hr using a peristaltic pump. Fractions were monitored for absorbance at 280 nm on a Beckman DU8B Spectrophotometer (Beckman, Irvine, California, U.S.A.).

**Protein-A-Sepharose CL-4B column chromatography.** Five ml of peak II from the Sephacryl S-200 column (Fig. 1A) was applied to a Protein-A-Sepharose CL-4B column (Pharmacia) equilibrated with 0.1 M sodium phosphate in 0.34 M NaCl, pH 7.0 (PBS). IgG bound to protein A was eluted from the column with 0.1 M glycine, pH 2.8. The IgG fraction was then dialyzed against 10 mM phosphate-buffered normal saline (PBS), pH 7.2.

**Rabbit anti-armadillo IgG (γ-chain-specific).** Fractions enriched for armadillo IgG by protein-A chromatography were reduced and heated (100°C for 3 min) in 0.1% SDS and 1% 2-mercaptoethanol and separated by SDS-PAGE (⁴) consisting of 4% stacking gel and 12% separating gel. One lane of the gel was stained with Coomassie brilliant blue R250 to locate the position of the immunoglobulin (Ig) heavy and light chains. The portion of the remaining unstained gel containing the heavy chains was removed, minced, and suspended in PBS. This suspension was injected subcutaneously into the foot pads and multiple sites on the backs of rabbits. The animals were given a booster injection 3 weeks later. The immune sera were collected 30 days after the primary injection.

**Purification of rabbit anti-armadillo γ-chain by affinity chromatography.** Ten ml of the rabbit anti-armadillo γ-chain serum was applied to an affinity column prepared with armadillo IgG bound to CNBr-activated Seoharose 4B (Pharmacia) using the

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¹ Wayne M. Meyers, M.D., Ph.D., kindly served as Editor in regard to the submission, review, revision, and acceptance of this manuscript.
² Received for publication on 19 February 1987; accepted for publication in revised form on 28 January 1988.
³ A. R. Vadiee, M.S.; E. J. Shannon, Ph.D.; T. P. Gillis, Ph.D., Immunology Research Department; R. C. Hastings, M.D., Ph.D., Chief, Laboratory Research Branch, GWL Hansen’s Disease Center, Carville, Louisiana 70721. U.S.A.
method described by the manufacturer. The rabbit anti-armadillo γ-chain-specific antibodies were eluted from the column with 0.1 M glycine, pH 2.8, and dialyzed against PBS.

Armadillo sera. Sera from nine-banded armadillos were collected approximately 15 months after the animals were injected intravenously with 6.9 × 10^8 viable M. leprae (Group I). These animals harbored an average of 3.3 × 10^9 M. leprae/g of liver tissue at necropsy. Sera were also collected from 4 nine-banded armadillos determined to be free of M. leprae infection after a 3-month quarantine period (Group II). Group II animals showed no signs of leprosy-like infection by routine screening, including detection of acid-fast bacteria (AFB) in ear biopsies and in blood smears.

Preparation of M. leprae extracts. M. leprae were provided by Dr. Patrick Brennan (NIH Contract #1AI-52582, Colorado State University) as irradiated, lyophilized bacilli purified from the lymph nodes of infected armadillos. M. leprae (10 mg dry weight) were suspended in 3 ml of PBS. The organisms were disrupted by sonication on ice for 30 min at 150 watts on a Sonifier Cell Disrupter with a temperature control module (Model W1851, Heat System; Ultrasonics, Inc., North Tonawanda, New York, U.S.A.). The sonicated material was centrifuged at 20,000 × g for 30 min at 4°C. The supernatant fraction has a protein concentration of 0.1 mg/ml (9) and was stored at −20°C until used.

Lipoarabinomannan (LAM). LAM derived from M. tuberculosis was provided by Dr. Patrick Brennan (Colorado State University).

Staining characteristics of M. leprae extracts. The supernatant extract of M. leprae was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were identified by staining with Coomassie brilliant blue R250 followed by silver staining. To identify carbohydrate moieties in extracts of M. leprae, gels were stained with a modified silver stain (10).

Immunoblot. Components separated by SDS-PAGE were electrophoretically transferred at constant voltage (10 V) for 18 hr to BA85 nitrocellulose paper (NCP) (Schleicher and Schuell, Inc., Keene, New Hampshire, U.S.A.) in a Tris-glycine-methanol buffer, pH 8.0 (11). After transfer, the NCP was reacted for 45 min in PBS containing 3% bovine serum albumin (BSA). The NCP was then incubated with an appropriate dilution of primary antibody for 1 hr at room temperature. The NCP was washed five times for 5 min each in PBS containing 0.05% Tween 20. It was then reacted with an appropriate dilution of secondary antibody, washed, and reacted with peroxidase-conjugated immunoglobulins. Next, the NCP was washed and developed with H_2O_2/horseradish peroxidase (HRP) color development reagent (Bio-Rad, Richmond, California, U.S.A.) for 10 min at room temperature as described by the manufacturer. The reaction was stopped by transfer of the NCP to 5% acetic acid in distilled H_2O.

125I-ConA binding of the M. leprae extracts. Concanavalin-A (ConA; Sigma Chemical Co., St. Louis, Missouri, U.S.A.) was radioiodinated by the Chloramine-T method (9). Free iodine was removed by gel filtration on P-6DG (Bio-Rad). M. leprae extracts were separated by SDS-PAGE and then electrophoretically transferred from the gel onto NCP. The NCP was incubated in a 1% BSA/PBS solution for 30 min at room temperature, and then incubated in 40 ml of 125I-ConA (1 × 10^6 cpm/ml) in 1% BSA/PBS for 1 hr at room temperature. The NCP was washed to remove unbound 125I-ConA, dried, and exposed to Kodak Ortho G film (Eastman Kodak Co., Rochester, New York, U.S.A.). Exposure of the NCP was at −70°C for 12 hr in an x-ray cassette fitted with a Coronex MRF 32 clear base intensifying screen (Dupont Co., Newton, Connecticut, U.S.A.).

RESULTS

Characterization of rabbit anti-armadillo γ-chain-specific serum. Armadillo serum proteins were fractionated on Sephacryl S-200, resolving the material into three distinct peaks (Fig. 1A). Peak II from the Sephacryl column was applied to a protein-A Sepharose CL-4B column and unbound material eluted with PBS, pH 7.2 (peak a, Fig. 1B). Protein-A bound material was eluted by the addition of low pH buffer (peak b).
and this material was analyzed by immunoelectrophoresis. A single immunoprecipitate band was observed when a sample of peak b was electrophoresed in agarose and then allowed to react with rabbit anti-whole armadillo serum (data not shown). The immune precipitate formed was localized in the zone characteristic for human IgG. SDS-PAGE analysis of peak b from the protein-A column (armadillo IgG) (Fig. 2, lane B) and human IgG (Fig. 2, lane C) showed identical patterns of migration for the reduced heavy chain molecule. In contrast, the major species of armadillo light chain (Fig. 2, lane B) was slightly higher in molecular weight ($M_r = 28,000$) as compared to those of human ($M_r = 25,000$) (Fig. 2, lane C). Rabbit antibodies reactive with the 51-kDa protein were purified by affinity chromatography using purified armadillo IgG as immunosorbent. Immunoreactivity of the purified antibodies located a single band at 51 kDa present in both purified armadillo IgG (Fig. 3, lane B) and whole armadillo sera (Fig. 3, lane C).

Staining and lectin binding characteristics of sonicated *M. leprae* supernate. Protein staining of SDS-PAGE profiles of *M. leprae* supernate with Coomassie blue followed by silver staining showed 10–12 bands with molecular weights from 12–72 kDa (Fig. 4, lane B). Periodate oxidation, followed by silver staining of the gel, greatly enhanced the carbohydrate-rich regions which appeared as diffuse-staining regions in the gel corresponding to 25 kDa, 33 kDa, and 66 kDa (Fig. 4, lane A). Further analysis of the carbohydrate staining components by lectin-binding studies indicated that ConA-reactive components were detected at regions corresponding to <10 kDa, 33 kDa, and 80 kDa (Fig. 5).

Antigenic components of *M. leprae* eliciting IgG or IgM antibodies. The supernatant of sonically disrupted *M. leprae* was fractionated by SDS-PAGE and then blotted to NCP. Blots were incubated with serum from *M. leprae*-infected armadillos (Group I) and developed with rabbit anti-armadillo $\gamma$-chain or rabbit anti-human $\mu$-chain-specific reagents (Fig. 6A). Armadillo IgG antibodies from most armadillos recognized antigens with molecular weights from 12–90 kDa (Fig. 6A, lane B). In contrast, when blots were developed using the $\mu$-chain-specific antisera, the majority of the armadillos tested showed a major diffuse band of immunoreactivity in the 33 kDa region (Fig. 6A, lane A). When normal armadillo sera (Group II) were analyzed, 2 of
Vadiee, et al.: M. leprae Ags Evoking Armadillo Abs

Fig. 2. Analysis of armadillo and human IgG by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were stained with Coomassie blue. Lane A = low molecular weight protein standards: phosphorylase b (92 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), lysozyme (14 kDa); lane B = purified armadillo IgG (0.9 μg); lane C = purified human IgG (1.1 μg).

4 animals showed IgG antibody reactivity to both the 33-kDa and 21-kDa antigen (Fig. 6B, lane B). When the same normal sera were analyzed for IgM antibody responses, all four animals showed immunoreactivity to the 33-kDa antigen (Fig. 6B, lane C).

IgG and IgM responses of M. leprae-infected armadillos to LAM. Lipoprotein-mannan was electrophoresed on SDS-PAGE and then blotted to NCP. Blots were incubated with serum from M. leprae-infected armadillos and developed with rabbit anti-armadillo γ-chain or rabbit anti-human μ-chain-specific reagent. When blots were developed for IgG activity, no sign of immunoreactivity was observed in any of the animals. However, when blots were analyzed for IgM activity, two diffuse bands of immunoreactivity were observed in the regions of approximately 50 kDa and 28–33 kDa (Fig. 7, lane B).

DISCUSSION
The mechanism by which M. leprae eludes the host defense system to cause disease is unknown. Since protection against disease...
is associated with an appropriate host immune response to *M. leprae*, delineation of the antigenic constitution of *M. leprae* is essential to the understanding of this process. This study was undertaken to characterize the antigenic components of *M. leprae* which evoked IgG or IgM antibody responses in normal and heavily infected armadillos. An antiserum specific to armadillo IgG (γ-chain) was prepared to identify the antigenic components of *M. leprae* which elicited IgG antibody responses in armadillos. IgM responses were detected using an anti-human μ-chain-specific reagent which has been shown to crossreact with armadillo IgM.

The IgG responses of heavily infected armadillos were directed at approximately 10 distinct bands with molecular weights from 33 kDa to 90 kDa and two additional bands at 12 kDa and 22 kDa. Chakrabarty, *et al.* (1) also reported immunoreactivity in heavily infected armadillos to the 12 kDa and 22 kDa antigen of *M. leprae*, but observed only four other major immunoreactive bands with molecular weights from 33 kDa to 67 kDa. It is possible that some of the additional immunoreactive components observed in our studies were degradative.
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Soluble extracts of M. leprae were separated by SDS-PAGE and electrophoretically blotted onto NCP. NCP was incubated with infected armadillo serum (1:50 dilution), washed, and treated with class-specific antisera (rabbit anti-armadillo γ-chain at 1:1000 dilution; rabbit anti-human μ-chain at 1:50 dilution). The strips were washed and treated with peroxidase-conjugated goat anti-rabbit immunoglobulin (IgA + IgM + IgG) at 1:1000 dilution. Visualization of antigen-bound antibody proceeded as explained in Figure 3. Lane A = reaction with rabbit anti-human μ-chain-specific antibody; lane B = reaction with rabbit anti-armadillo γ-chain-specific antibody; lane C = low molecular weight markers.

One explanation for the differences in antibody responses seen between the two studies is the likely individual variations in antibody responses among the highly outbred population of armadillos. Alternatively, recognition of larger numbers of immunoreactive bands could be due to a potential increase in sensitivity and specificity of our assay using isotype-specific reagents, as opposed to radiolabeled protein-A.

Furthermore, the above pattern of IgG immunoreactivity may reflect the relative immunodominance or accessibility of these proteins in the intact bacillus, or their rel-
Fig. 7. Immunoblot to demonstrate IgM antibody response of infected armadillos to lipoarabinomannan (LAM). LAM (80 μg ml) was separated by SDS-PAGE and electrophoretically blotted onto NCP. (Technical procedure is same as in Fig. 6A.) Lane A = low molecular weight markers; lane B = reaction with rabbit anti-human μ-chain-specific antibody.

ative concentration in the bacterial cell sonicate. Similarities between human and armadillo antibody responses have been reported by noting immunoreactivity to M. leprae components with molecular weights of 12, 22, 28, 36, 41, and 86 kDa.

Comparison of immunoblot analyses of armadillo IgG responses to M. leprae components with that of humans indicates that M. leprae-infected armadillos generally recognize and produced antibodies to a larger number of M. leprae components than multibacillary patients. For example, analysis of sera from M. leprae-infected armadillos indicated that, in addition to the above immunoreactive components recognized by human sera, armadillo sera reacted to a group of M. leprae components with molecular weights ranging from 45 kDa to 90 kDa and, among these, only the 86 kDa component of M. leprae was recognized by human sera (17). Our results support earlier findings that armadillos produce antibody to a great diversity of M. leprae antigens as opposed to the previously reported limited repertoire produced by Hansen's disease (HD) patients. Differences in antibody profiles between experimental M. leprae-infected armadillos and humans afflicted with HD could be due to the effect of chemotherapy of patients as suggested by Klatser, et al. (17), or as a result of immune recognition of various antigens governed by immune response genes.

The predominant IgM antibody responses of the majority of infected armadillos were to a component which migrated as a broad diffuse band with a molecular weight of approximately 28–33 kDa. Staining characteristics using a modified silver stain for carbohydrates and the ability of the 33 kDa antigen to bind 125I-ConA suggested that this molecule is composed of carbohydrates containing mannose or glucose residues. Additionally, when we analyzed the IgG and IgM antibody responses of an infected armadillo to the highly cross-reactive mycobacterial antigen LAM (17) derived from M. tuberculosis. IgM activity was observed only to two broad diffuse bands at the regions of 28–33 kDa and 50 kDa. Comparison of the SDS-PAGE migration patterns and antibody binding characteristics of the 33 kDa material with that of purified M. tuberculosis LAM (17) suggested that
the two components were chemically and immunologically related.

The ability of a 33 kDa molecule to elicit a strong antibody response in armadillos and humans infected with *M. leprae* has been reported by Chakrabarty, *et al.* (1). However, they reported no reactivity to 33 kDa in normal armadillos and uninfected humans by immunoblot analyses. These investigators used 125I-Staph-A protein, which detects primarily IgG antibodies. In our studies, the use of isotypic-specific antisera demonstrated the presence of IgG and primarily IgM antibodies to the 33-kDa antigen of *M. leprae* in sera of normal and infected animals. The immunoreactivity observed in sera from normal armadillos may indicate previous exposure of these armadillos to *M. leprae* or to a crossreactive mycobacterial antigen present in the environment. In addition, since the 33-kDa antigen seems to elicit primarily IgM-type responses, the use of 125I-Staph-A protein may not always be sufficient for detection of antibodies to this antigen. *M. leprae* antibodies. This further supports the need for analysis of antibody responses to *M. leprae* using isotype-specific reagents to obtain a more precise understanding of the humoral response during infection.

Further studies in the armadillo relating immune response to defined antigens with disease status should strengthen our understanding of the infection in the armadillo model for HD and, potentially, natural infection with *M. leprae* in man.

**SUMMARY**

Armadillo IgG and IgM antibody responses to *Mycobacterium leprae* were analyzed using isotypic-specific antisera by means of immunoblotting. Blots developed for IgG antibodies to *M. leprae* showed multiple protein antigens (Mr = 12–90 K) in some heavily infected armadillos. In contrast, blots developed for IgM antibodies to *M. leprae* showed a single, broad, diffuse band of immunoreactivity at approximately 33 kDa. The 33-kDa immunogen was detectable with silver stain modified for carbohydrate reactivity, suggesting the presence of a polysaccharide component. In addition, binding of 125I-concanavalin A to the 33-kDa component demonstrated the presence of mannose and/or glucose residues.

**RESUMEN**

Se analizaron las respuestas de armadillos en anticuerpos IgG e IgM anti- *Mycobacterium leprae* usando antisueros específicos para isotipos e inmunoelectrotransferencia. Cuando el revelado se hizo para anticuerpos IgG contra *M. leprae*, se encontraron múltiples componentes antitóxicos (Mr = 12–90 K) en algunos armadillos muy infectados. En contraste, cuando el revelado se hizo para anticuerpos IgM contra *M. leprae*, se encontró una sola banda amplia y difusa de inmunoreactividad de aproximadamente 33 kDa. El immunógeno 33 kDa fue detectado con tinción de plata modificada para carbohidratos, sugiriendo la presencia de un componente polisacárido. Además, el enlazamiento de concanavalina A-125I al componente 33 kDa demostró la presencia de residuos de manosa y/o glucosa.

**RÉSUMÉ**

On a analysé chez le tatou les réponses en anticorps IgG et IgM à *Mycobacterium leprae*, en utilisant des échantillons d'antisérums isotypiques spécifiques, au moyen d'une technique de détection par transfert (immunoblotting). Les transferts d'anticorps IgG contre *M. leprae* montraient des antigènes protéiques multiples (Mr = 12–90 K) chez quelques tatous fortement infectés. Par contre, les transferts (blots) mis en évidence pour les anticorps IgM contre *M. leprae*, n'ont révélé qu'une bande unique, large et diffuse, d'inmunoreactivité à environ 33 kDa. L'immunogène 33 kDa pouvait être décelé avec une coloration argentique modifiée pour la réactivité carbohydatée, ce qui suggère la présence d'un constituant polysaccharidique. De plus, la liaison de la 125I-concanavaline A au constituant de 33 kDa témoignait de la présence de résidus de manose, de glucose, ou de ces deux sucrés à la fois.

**Acknowledgments.** This work was supported in part by grants from American Leprosy Missions, Inc., and the Hansen’s Disease Foundation, Inc. We also thank Dr. R. N. Mshana for helpful suggestions in the text.

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CHAPTER II  Armadillo IgG and IgM antibody responses to phenolic glycolipid-I during experimental infection with M. leprae.
Dear Dr. Hastings:

I am in the process of writing my dissertation which will partially fulfill the requirements for the degree of Doctor of Philosophy in the Department of Microbiology at Louisiana State University in Baton Rouge. With your permission, I would like to include a reprint of the article entitled:

"Armadillo IgG and IgM Antibody Responses to Phenolic Glycolipid-I During Experimental Infection with M. leprae".

By Abdul R. Vadiee, et al.,

which appeared in:


The bound dissertation and a microfilmed copy will become part of the collection of the university library. Your attention to this matter is greatly appreciated. Thank you very much.

Sincerely,

Abdul R. Vadiee
Armadillo IgG and IgM Antibody Responses to Phenolic Glycolipid-I During Experimental Infection with *M. leprae*


The armadillo, when experimentally inoculated with *Mycobacterium leprae*, develops a disseminated infection similar in many respects to human lepromatous leprosy (6-7). In serological studies of leprosy, armadillos have advantages over human subjects in that a number of armadillos can be inoculated at the same time with known quantities of *M. leprae* and their serological responses can be studied over time in the absence of drug therapy. However, studies related to the immunological responses of *M. leprae*-infected armadillos have been few and, to date, we do not have a concise understanding of the humoral antibody responses of armadillos to *M. leprae*.

Using a radioimmunoassay, Harboe, et al. (3) demonstrated increased amounts of antibodies to *M. leprae* antigen 7 during the course of experimental infection. In immunoblotting studies using 125I-labeled protein A, Chackrabarty, et al. (4) have shown the presence of antibody to several antigenic components of *M. leprae* in the sera of infected armadillos. Recently, Truman, et al. (10) have shown that estimations of armadillo IgM antibodies to an *M. leprae*-specific antigen, phenolic glycolipid-I (PGL-I) (3), can be useful in following the course of the infection.

In any infectious disease, one would also be interested in an analysis of the IgG responses during the course of the disease process. A limitation in the study of IgG responses of the armadillo has been the unavailability of appropriate reagents. To detect armadillo IgG antibody, we prepared an antibody with specificity to armadillo γ-chain (Vadiee, A. R., Master's thesis, Southeastern Louisiana University, 1985). With the availability of an anti-armadillo γ-chain-specific reagent and μ-chain-specific, crossreactive, anti-human IgM antibody (10), plasma samples from 11 armadillos collected during the course of infection were analyzed using ELISAs to assess the IgG and IgM responses to PGL-I.

**MATERIALS AND METHODS**

**Animals.** Nine-banded armadillos (*Dasyopus novemcinctus*) were captured within a 30-mile radius of the Gillis W. Long Hansen's Disease Center (GWLHDC), Carville, Louisiana, U.S.A. The animals were screened for wild-type infection with mycobacteria once a month for 3 months by examining the buffy coats of peripheral blood and ear snips for the presence of acid-fast bacilli (AFB). Animals found to be negative for AFB were then inoculated with *M. leprae*. Eleven armadillos were inoculated intravenously with 5 × 10⁸ armadillo-passage *M. leprae*. Plasma samples were collected prior to inoculation (day 0) and at approximately 3-month intervals for a period of 1 year. The samples were stored at −20°C until tested.

The 11 animals were inoculated for the purpose of *M. leprae* production. The 3-month sampling frequency is routine and is based upon the likelihood that more fre-
quent sampling might kill the animals prematurely. These animals were sacrificed when they exhibited a heavy dissemination of *M. leprae* infection based on histological examination of ear biopsies. Plasma samples were analyzed up to approximately 1 year after inoculation.

**Enzyme-linked immunosorbent assay (ELISA).** Armadillo IgG and IgM antibodies to PGL-I were detected using a modification of the method of Cho, *et al.* (2). Armadillo-derived PGL-I, kindly provided by Dr. P. Brennan (NIH contract #AI-52582), was suspended in 0.05 M carbonate-bicarbonate, pH 9.2 coating buffer by sonication for 30 sec at 70 watts using a sonifer cell disrupter with a temperature control module (Model W1851; Heat System Ultrasonic, Inc., North Tonawanda, New York, U.S.A.). The suspension was diluted to contain 40 μg of PGL-I per ml in coating buffer. Fifty μl of the PGL-I suspension was added to each of 48 wells of a 96-well, polyvinyl chloride, flat-bottomed microtiter plate (Cook Labs., Alexandria, Virginia, U.S.A.); the remaining 48 wells received 50 μl of the coating buffer. The plates were incubated at 4°C overnight. The wells were washed three times with 200 μl of 0.01 M phosphate buffered saline (PBS), pH 7.2, containing 1% bovine serum albumin (BSA), and the wells were blocked by incubation with 100 μl of PBS containing 5% BSA at room temperature (RT) for 1 hr. The contents were aspirated, 50 μl of the armadillo plasma diluted (1:250) in 1% BSA-PBS was added to all wells, and the plates were incubated at RT for 1 hr.

Attempts at directly conjugating peroxidase molecules to rabbit anti-armadillo γ-chain proved unsatisfactory. Therefore, for detection of armadillo IgG antibodies to PGL-I, 50 μl of rabbit anti-armadillo IgG (γ-chain specific) diluted 1:1000 in 1% BSA-PBS was added subsequent to the primary antibody and washing steps. The plates were incubated at RT for 1 hr. After washing, 50 μl of peroxidase-conjugated goat anti-rabbit IgG (Cappel Laboratories, Downington, Pennsylsvania, U.S.A.) containing 0.02% H₂O₂ in 0.02 M sodium acetate buffer, pH 5.5, was added to each well. The plates were incubated at RT for 10 min, and the reaction was stopped by adding 5 N HCl (50 μl/well). Absorbance at 492 nm was read with a spectrophotometer (Titertek Multiscan; Flow Laboratories, Richmond, Virginia, U.S.A.).

An indirect ELISA for measuring armadillo IgM resulted in an unacceptably high background. Therefore, to detect armadillo IgM antibodies to PGL-I, 50 μl of peroxidase-conjugated rabbit anti-human IgM (μ-chain specific; Dako Corp., Santa Barbara, California, U.S.A.), diluted at 1:400 in 1% BSA-PBS, was added per well and incubated for 1 hr. The IgM immunoreactivity was detected as explained above. The antibody reactivity to PGL-I for each plasma sample was calculated by subtracting the mean absorbance of duplicate samples in the wells coated with carbonate buffer from the mean of duplicates in the PGL-I-coated wells.

**Statistical analysis.** Results were analyzed for statistical significance on a Hewlett-Packard 984SB computer using the Pearson correlation coefficient test and the two-tailed paired *t*-test. Values of *p* < 0.05 were considered to be statistically significant.

**RESULTS**

The evolution of IgG and IgM anti-PGL-I responses among this group of 11 armadillos is shown in Figures 1 and 2. Compared to baseline, the IgG and IgM anti-PGL-I increased significantly by 97 days postinoculation. There were significant progressive increases in the absorbance values up to 272 days.

With the exception of animal 383, IgM anti-PGL-I absorbances were relatively homogeneous (Fig. 3). On the other hand, the IgG anti-PGL-I responses of individual animals showed marked variations (Fig. 4). Beginning from day 272 postinoculation, the animals could be divided into two groups based on their IgG anti-PGL-I absorbance values. Animals in group A had high absorbance values (>0.7). The other animals, designated group B, maintained an IgG anti-PGL-I absorbance value of <0.7. In addition, the IgG anti-PGL-I absorbance value...
DHYS POST INOCULATION

Fig. 1. IgG anti-PGL-I mean ± S.E.M. absorbance values of 11 armadillos during course of experimental infection. Asterisks indicate values significantly different from preceding values (paired t-test).

decreased dramatically among most animals in group B during the terminal stage of the disease.

These armadillos were also regularly examined histologically for the presence of AFB in ear biopsies as a means of detecting dissemination of the disease. By this criterion, group B animals developed disseminated disease earlier than group A (288 days vs 399 days) and had a shorter mean survival time (422 days vs 735 days) (The Table). However, these differences between the two groups were not statistically significant.

DISCUSSION

The observations made on IgM anti-PGL-I parallel the findings reported by Truman, et al. (10) that *M. leprae* infection elicits a prolonged IgM response to PGL-I in armadillos.

IgG anti-PGL-I also showed a significant increase with time. Although there was considerable individual variation, a sharp decrease in the level of IgG anti-PGL-I absorbance values was seen among some of the animals during the terminal stage of the disease (Fig. 4: group B, 363 days post-inoculation). While the reason for this sudden decrease is unclear, one possibility could be the presence of a high concentration of antigen in the sera with the formation of immune complexes during the terminal stage of the disease.

Previous studies have shown that armadillos less susceptible to infection with *M. leprae* exhibit a positive lepromin skin test (5). These animals also showed a positive skin test in response to PGL-I, suggesting that PGL-I preparations are capable of eliciting a T-cell response in the armadillo. In human studies, lymphocyte proliferation to PGL-I was shown to be positively correlated with IgG antibodies to PGL-I (7), also implying that T cells are involved in the response to PGL-I. Furthermore, Levis, et al. (9) have reported high IgG anti-PGL-I in some patients with tuberculoid (BT) leprosy, a form of leprosy associated with relative control of the infection.

We also observed a group of armadillos (Fig. 4: group A) with high absorbance values for IgG anti-PGL-I which appeared to be able to delay the dissemination of *M. leprae* infection as compared to animals with

<table>
<thead>
<tr>
<th>Armadillo group</th>
<th>IgG ELISA at 365 days post-inoculation</th>
<th>Longevity (days)</th>
<th>Time of appearance of AFB in ear biopsy (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (N = 5)</td>
<td>&gt;0.7</td>
<td>735 ± 162</td>
<td>399 ± 61</td>
</tr>
<tr>
<td>B (N = 6)</td>
<td>&lt;0.7</td>
<td>422 ± 37</td>
<td>288 ± 26</td>
</tr>
</tbody>
</table>

The Table. Comparison of IgG absorbance values to PGL-I with longevity and time until the appearance of AFB in ear biopsies among 11 armadillos inoculated with *M. leprae*.
low absorbance values. These findings suggest that the ability to control infection with *M. leprae* in both armadillos and leprosy patients may be associated with their ability to mount an IgG antibody response to PGL-I.

While data reflecting antibody responses to PGL-I, including ours, are presented as absorbance values, these values do not directly reflect absolute values for IgG or IgM antibodies. Antibodies to *M. leprae* are not expected to be protective in leprosy since *M. leprae* is an intracellular organism, but production of IgG anti-PGL-I antibodies might be a reflection of more competent T-cell function than the production of IgM antibodies alone. Cho, *et al.* (7) were the first to suggest that the high levels and the persistence of IgM antibody to PGL-I in lepromatous patients may be due to a lack of T-helper cell-mediated switch from IgM to IgG antibody. Levis, *et al.* (8) and Koster, *et al.* (9) have proposed that a significant IgG anti-PGL-I response among paucibacillary leprosy patients may be due to successful T-cell help for an IgM to IgG switch. Thus, there may be an association between elevated IgG anti-PGL-I responses and the T-cell immunological recognition of *M. leprae*.

Finally, the availability of a serological test capable of distinguishing armadillos with various degrees of susceptibility to *M. leprae* along with histological examination of tissue biopsies for the determination of disease progression offers opportunities for the management of *in vivo* production of *M. leprae*. It also could help in the understanding of possible differences in the immunological capability of less susceptible animals (those capable of delaying the onset of dissemination of infection) from the more susceptible animals. The IgG anti-PGL-I assay might be one such test.

**SUMMARY**

The kinetics of antibody responses of *Mycobacterium leprae*-infected armadillos to phenolic glycolipid-I (PGL-I) were studied by means of ELISA. The levels of both IgG
and IgM antibodies to PGL-1 increased with time. Some animals were less susceptible to disseminations of *M. leprae* infection and lived longer than others. These animals had high absorbance values (>0.7) for IgG anti-PGL-1 compared to more susceptible armadillos that had lower absorbance values for IgG anti-PGL-1.

RESUMEN

Utilizando un inmunoenzima test (ELISA) se estudió la cinética de la respuesta en anticuerpos contra el glicolipido fenólico-1 (GLF-1) en armadillos infectados con *Mycobacterium leprae*. Los niveles de anticuerpos IgG e IgM aumentaron con el tiempo. Algunos animales fueron menos susceptibles a la infección diseminada con *M. leprae* y vivieron más que otros. Estos animales mostraron valores más altos de absorbancia (>0.7) para IgG anti-GLF-1 que los armadillos más susceptibles.

RéSUMÉ

La cinétique de la réponse en anticorps au phénoglycolipide-1 (PGL-1) chez des tatous infectés par *M. leprae*, a été étudiée au moyen d’une épreuve ELISA. Les taux d’anticorps IgG et IgM au PGL-1 ont augmenté avec le temps. Certains animaux étaient moins susceptibles à la dissémination de l’infection par *M. leprae*, et ont survécu plus longtemps que les autres. Ces animaux témoignaient de valeurs d’absorption élevées (>0.7) pour les IgG contre PGL-1, par rapport aux tatous plus susceptibles qui présentaient des valeurs d’absorption plus faibles pour ces anticorps.

Acknowledgments. This study was supported in part by grants from the American Leprosy Missions (ALM) and the Hansen’s Disease Foundation. The authors wish to express their appreciation to personnel of the Microbiology Research Department for making available the armadillo serum samples. We are also grateful to Mr. Mel Morales for the computer analyses, Mr. Sterling Christy for processing and storing the serum samples, and to Ms. Penne Cason for clerical assistance.

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**Vadiee, et al.: Armadillo IgG and IgM Ab to PGL-I**


CHAPTER III  The evolution of antibody response in armadillos inoculated with _M. leprae_.

Submitted for publication to the _Journal of Leprosy Review_
ABSTRACT

Plasma from 30 armadillos (Dasypus novemcinctus) was collected prior to inoculation and at approximately 3-month intervals for a period of 1-3 years. These animals were inoculated intravenously with $6.1 \times 10^8 \pm 2 \times 10^8$ (x + S.D.) armadillo-derived M. leprae. These samples were analyzed for antibodies of IgM and IgG class to phenolic glycolipid-I (PGL-I) and to sonicated M. leprae components using ELISA and immunoblotting techniques, respectively. We had previously observed among a group of 11 armadillos, that some animals produced and maintained a high IgG antibody response to PGL-I. In this study, an animal's ability to produce and maintain an elevated IgG anti-PGL-I response was significantly correlated with their ability to delay dissemination of the infection and their ability to survive longer. When the animals were moribund, a significant decrease in the IgG anti-PGL-I absorbance value was observed. The detection of PGL-I in the plasma samples collected from moribund armadillos suggested that high concentrations of PGL-I in the plasma may have contributed to a drop in absorbance values by the formation of non-lattice-type immune complexes in vivo.

As detected by immunoblotting, the IgM and IgG response to antigens derived from sonically disrupted M. leprae was directed toward molecules with broad bands of immunoreactivity ranging from 21-45-kDa. There were no distinguishing features of these antibody responses among armadillos as was evident with the IgG anti-PGL-I responses.
INTRODUCTION

Leprosy in man has a broad clinical manifestation determined by the host parasite relationship. The spectrum of variation in resistance to infection with Mycobacterium leprae (M. leprae) ranges from highly resistant (tuberculoid leprosy) to highly susceptible (lepromatous leprosy)(19). Nine-banded armadillos (Dasypus novemcinctus) are highly susceptible to infection with M. leprae (15) and when inoculated, they develop primarily lepromatous-type disease (14). Therefore, armadillos are considered by many investigators to be a good experimental model for studying lepromatous leprosy. One characteristic of human lepromatous leprosy, as well as of infected armadillos, is the production of large amounts of antibodies against mycobacterial antigens (2,21). These antibodies have played a significant role in describing the immunogenic structure of M. leprae. Components such as antigen 7 (9), cell wall polysaccharides (2), proteins (2,18) and phenolic glycolipid-I (PGL-I)(6,20,22) are recognized as antibody evoking immunogens in both humans and armadillos.

Due to difficulties in detection of subclinical leprosy in man, a description of the evolution of antibodies to components of M. leprae among patients in the presence or absence of chemotherapy has not been reported. Armadillos experimentally inoculated with M. leprae offer an opportunity to describe the evolution of antibodies to M. leprae following exposure to known quantities of M. leprae and in the absence of chemotherapy.
This study describes the evolution of armadillo IgM and IgG antibodies to PGL-I and sonicated M. leprae using ELISA and immunoblotting.
MATERIALS AND METHODS

Armadillo plasma: Plasma from 30 nine-banded armadillos was collected prior to inoculation (day 0) and at approximately 3-month intervals for a period of 1-3 years. The conditions for adopting the animals into the colony and their maintenance have been described previously (21). These animals were inoculated intravenously with \(6.1 \times 10^8 + 2 \times 10^8\) (x + S.D.) armadillo-derived \(M. lepra\).

Enzyme-linked immunosorbent assay (ELISA): One hundred ninety-two plasma samples were analyzed by means of ELISA. The ELISA, for detection of armadillo IgM and IgG antibodies to PGL-I, was carried out as described previously (22). Armadillo-derived PGL-I was provided by Dr. Patrick Brennan (NIH Contract #AI-52582, Colorado State University).

Preparation of \(M. lepra\) extracts: \(M. lepra\) were provided by Dr. Patrick Brennan (NIH Contract #AI-52582, Colorado State University) as irradiated, lyophilized bacilli purified from the lymph nodes of infected armadillos. \(M. lepra\) (10 mg dry weight) was suspended in 3 ml of 0.01 M phosphate buffered saline (PBS) pH 7.0. The organisms were disrupted by sonication on ice for 30 min at 150 watts on a sonifier cell disrupter with a temperature control probe (Model W1851, Heat System, Ultrasonics, Inc., North Tonawanda, New York, USA) adjusted for 8° C. The sonicated material was aliquoted and stored at -20° C until used.

Immunoblot: \(M. lepra\) components (3.33 mg/ml) were separated by SDS-PAGE and electrophoretically transferred at constant voltage (10 V) for 18 hr to BA 85 nitrocellulose paper (NCP) (Schleicher and Schuell, Inc., Keene, New Hampshire, USA) in a tris-glycine-methanol
buffer, pH 8.0 (16). After transfer, the NCP was incubated at room temperature (RT) for 45 min in 0.01 M PBS containing 3% bovine serum albumin (BSA). The NCP was then incubated with armadillo plasma at 1:50 dilution for 1 hr at RT. The NCP was washed five times for 5 min each in 0.01 M PBS containing 0.05% Tween 20. It was then reacted with rabbit anti-armadillo gamma-chain at 1:1000 dilution or with rabbit anti-human u-chain at 1:50 dilution. The NCP strips were washed and treated with peroxidase conjugated goat anti-rabbit immunoglobulins (IgM + IgG + IgA) (Cappel Laboratories, Downingtown, Pennsylvania, USA) at 1:1000 dilution. Next, the NCP was washed and developed with \( \text{H}_2\text{O}_2 \)/horseradish peroxidase (HRP) color development reagent for 10 min at RT as described by the manufacturer (Bio-Rad, Richmond, California, USA). The reaction was stopped by transfer of the NCP to 5% acetic acid in deionized \( \text{H}_2\text{O} \).

**Extraction of PGL-I from armadillo plasma:** Plasma samples (0.3 - 0.5 ml) were added dropwise to 5 ml of 95% ethanol, resulting in the formation of a whitish precipitate. The samples were centrifuged at 1380 x G for 10 min and the supernatants were decanted. The excess ethanol was removed by inverting the tubes and allowed to drain for 10 min. Lipids were extracted from the residue with 5 ml chloroform:methanol (2:1 V/V) at 50° C overnight. The extracts were centrifuged at 1380 x G for 10 min. The supernatants were removed and residues were washed with 5 ml of chloroform:methanol (2:1) and re-centrifuged. The two supernatants from each sample were combined and taken to dryness at 50° C under nitrogen. The procedure for extracting the PGL-I from the total lipid extract was based on that of Hunter, et al. (10). Briefly, dried lipids were dissolved in
chloroform and applied to a silicic acid:celite column (2:1 V/V). The column was successively eluted with two bed volumes each chloroform, 2% methanol in chloroform and 5% methanol in chloroform. The 2% and 5% eluates were combined and taken to dryness under nitrogen at 50° C and analyzed for PGL-I by thin-layer chromatography (TLC) and ELISA.

For TLC, samples were applied to high performance silicel gel plate (Sigma, St. Louis, MO) and run in a solvent system composed of ether:acetone (3:2, V/V). The plates were air dried and PGL-I was located by spraying the plates with orcinol:sulfuric acid reagent and heating in a drying oven at 110° - 115° C for 3-5 min. PGL-I at 5-10 ug served as the standard marker.

For ELISA, the samples were suspended in 100 ul of 0.05 M carbonate-bicarbonate buffer, pH 9.2. The samples were further diluted 1:4 in the carbonate-bicarbonate buffer and were used for coating the microtiter plate (50 ul/well). The plate was incubated at 4° C overnight. The wells were washed 3x with 200 ul of 0.01 M PBS pH 7.2, containing 1% BSA, and blocked by incubation with 100 ul of 0.01 M PBS containing 5% BSA at RT for one hr. The contents were aspirated and 50 ul of monoclonal antibody F8b4 (kindly provided by Thomas Buchanan) diluted 1:500 in 1% BSA/PBS was added to all wells. The plate was incubated at RT for one hr. After washing, 50 ul of peroxidase-conjugated rabbit anti-mouse IgM (u-chain-specific) (Cappel Laboratories, Downington, Pennsylvania, USA) at 1:500 dilution was added per well and incubated for one hr at RT. After washing the plate, 50 ul of 0.04 mg/ml solution of ortho-phenylene diamine (Sigma Chemical Co., St. Louis, MO, USA) containing 0.02% H2O2 in 0.02 M
sodium acetate buffer, pH 5.5, was added to each well. The plate was incubated at RT for 10 min, and the reaction was stopped by adding 5 M HCl (50 ul/well). Absorbance at 492 nm was read with a spectrophotometer (Titertek Multiscan, Flow Laboratories, Richmond, Virginia, USA). Wells not coated with antigen served as negative control. For positive controls, 50 ul of known concentrations of PGL-I ranging from 0.025 to 80 ug/ml were used for coating the wells.

Statistical analysis: Results were analyzed for statistical significance on a Hewlett-Packard 9845T computer using the one tailed paired t-test and t-test. Values of p < 0.05 were considered to be statistically significant.
RESULTS

Armadillo antibodies to PGL-1: The evolution of IgM and IgG anti-PGL-I response among a group of 30 armadillos is shown in Figure 1. Compared to the baseline (0 day), the anti-PGL-I response increased significantly up to approximately 450 days post inoculation (PI) for IgM and up to approximately 630 days PI for IgG. The IgG anti-PGL-I responses of individual animals appeared to be heterogeneous and in general two patterns of responsiveness to PGL-I were observed as early as 190 days PI (Figure 2, Groups A and B). The animals in Group A had a significant high IgG anti-PGL-I absorbance value throughout the course of experimental infection. Furthermore, a comparison of IgG anti-PGL-I absorbance value among animals in Groups A and B at 363 days PI, when all animals in both groups were still alive, showed a statistically significant difference in their absorbance values (Table 1).

When animals in Group A were compared to those in Group B in relation to their longevity after inoculation with M. leprae, it was shown that animals in Group A survived for a longer period (1051 days vs 563 days) (Table 1). The armadillos used for this study were also regularly examined histologically for the presence of acid fast bacilli (AFB) in ear biopsies as a means of detecting dissemination of the disease. By this criterion, Group A animals delayed the dissemination of the disease as compared to those in Group B (717 days vs 417 days). These differences as represented in Table 1 were statistically significant (p < 0.01).

The animals from both groups were sacrificed when they exhibited a heavy dissemination of M. leprae infection as determined by
histological examination of their ear biopsies. Interestingly, the bacterial load in organs like the spleen, liver and lymph nodes in both populations was quite similar. However, one animal from Group A is still alive and information regarding its bacterial load is not available.

Armadillo antibodies to M. leprae components: Plasma samples collected during the first year post inoculation with M. leprae from a total of 9 animals, 4 from Group A and 5 from Group B (Figure 2), were selected and used in immunoblot analysis. The results obtained from both groups indicated a predominant IgM and IgG antibody response to be directed toward M. leprae components with molecular weights ranging from 21 - 45-kDa. These components were also reactive with antibody of the IgM and IgG class in normal plasma (0 day) in 8/9 and 2/9 armadillos, respectively. Representative data as derived by selection of a given animal from each group is presented in Figure 3. One unique armadillo from Group B produced antibodies of IgM and IgG class to multiple components of M. leprae with time post-inoculation (Figure 4). Multiple bands of immunoreactivity ranging from approximately 5-kDa to 92-kDa were observed when blots were developed for IgG response using a plasma sample collected at 363 days PI. IgM response appeared as broad diffuse bands of immunoreactivity. IgM response to the 65-kDa protein was observed among most animals throughout the course of infection. IgG response to the 65-kDa protein was mostly observed during the later phase of infection. The animal with antibody activity to multiple components of M. leprae showed a very strong IgG response to the 65-kDa protein (Figure 4).
Analysis of components extracted from armadillo plasma by TLC and ELISA: Eight armadillos, 3 from Group A and 5 from Group B were selected for this phase of the study. Plasma samples of each animal were selected at 4 periods and analyzed for presence of PGL-I. These included the plasma collected prior to inoculation with *M. leprae* (normal) and 3 which were collected during the early, mid and late phase of infection. Seven out of the 8 plasma samples which were collected during the latter phase of infection contained components which had migration patterns similar to that of standard PGL-I using TLC. These included 2 animals from Group A (animal #1 and #2) and 5 animals from Group B (animals #4-8) (Table 2). Furthermore, plasma samples of 2 animals in Group B (animal #5 and #6) which were collected during the mid-phase of infection also had components with migration patterns similar to that of PGL-I (Table 2). A representative migration pattern of samples collected during the latter phase of infection from animals in Group A and B using TLC is shown in Figure 5. A broad staining band was observed at the region characteristic to that of standard PGL-I. It is clear that the RF value of tested samples are slightly smaller than that of standard PGL-I. However, it is believed that the discrepancy may be due to the association of other components with PGL-I. The TLC results for each plasma sample were visually scored and were presented in Table 2.

To confirm that the components extracted from the plasma were PGL-I, we analyzed their immunoreactivity in ELISA by using a specific monoclonal antibody F8b4 to PGL-I. The samples having migration patterns similar to that of PGL-I were shown to be reactive
with MAB F8b4 and, therefore, identified as PGL-I (Table 2). A sample collected during the early phase of infection from animal #5 was positive for PGL-I in ELISA, but not TLC.
Figure 1. IgM and IgG anti-PGL-I response of 30 armadillos during the course of experimental infection. Asterisks indicate IgG anti-PGL-I absorbance values significantly different from preceding absorbance values (paired t-test).
**RBS (492 nm) PGL-1**

- **IgG (mean ± sem)**
- **IgM (mean ± sem)**

**PAIRED t (N)**
- *p < 0.05
- **p < 0.01
- ***p < 0.001

**Days Post Inoculation with M. leprae**

<table>
<thead>
<tr>
<th>Days</th>
<th>IgG (mean ± sem)</th>
<th>IgM (mean ± sem)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>(29)</td>
<td>(30)</td>
</tr>
<tr>
<td>180</td>
<td>(30)</td>
<td>(14)</td>
</tr>
<tr>
<td>270</td>
<td>(30)</td>
<td>(8)</td>
</tr>
<tr>
<td>360</td>
<td>(8)</td>
<td>(9)</td>
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<tr>
<td>450</td>
<td>(8)</td>
<td>(4)</td>
</tr>
<tr>
<td>540</td>
<td></td>
<td></td>
</tr>
<tr>
<td>630</td>
<td></td>
<td></td>
</tr>
<tr>
<td>720</td>
<td></td>
<td></td>
</tr>
<tr>
<td>810</td>
<td></td>
<td></td>
</tr>
<tr>
<td>900</td>
<td></td>
<td></td>
</tr>
<tr>
<td>990</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. Kinetics of IgG anti-PGL-I mean + S.E.M. animals in group A (n=11); animals in group B (n=19).
ABS(492nm) PGL-1

GROUP A

GROUP B

DAYS POST INOCULATION WITH M. leprae
Figure 3. Demonstration of antigenic components of *M. leprae* eliciting IgM and IgG antibody response. A representative immunoblot characteristic common to animals in group A and group B are shown here. Sonicated *M. leprae* components were separated by SDS-PAGE and electrophoretically blotted onto NCP. NCP was incubated with armadillo serum (1:50 dilution), washed, and treated with class-specific antisera (rabbit anti-armadillo gamma-chain at 1:1000 dilution; rabbit anti-human and mu-chain at 1:50 dilution). The NCP strips were washed and treated with peroxidase-conjugated goat anti-rabbit immunoglobulin (IgM + IgG + IgA) at 1:1000 dilution.
group B

IgG

IgM

0 97 272 363 453
DAYS

45 KD

31 KD

21 KD

0 97 272 363 453
DAYS
Figure 4. Demonstration of antigenic components of *M. leprae* eliciting IgM and IgG antibody response. Technical detail is similar to that of Figure 3.
Figure 5. Comparison of the migration pattern of the component extracted from armadillo plasma with that of a standard PGL-I, using TLC. Samples were applied to high performance silicel gel plate and run in a solvent system composed of ether:acetone (8:2, v/v). Samples were located using orcinol:sulfuric acid reagent. Lanes 1–4, components extracted from plasma of 4 armadillos during the latter phase of infection.
## TABLE 1

Comparison of IgG absorbance values to PGL-1 with longevity and time until the appearance of AFB in ear biopsies among 30 armadillos inoculated with *M. leprae*

<table>
<thead>
<tr>
<th>Armadillo Group</th>
<th>IgG ELISA at 360 Days Post Inoculation (OD ≥ 492nm)</th>
<th>Longevity (Days)</th>
<th>Time to AFB Appearance in Ear Biopsy (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High IgG Anti-PGL-1 Responder (N=11)</td>
<td>0.745 ± 0.068&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1051 ± 65</td>
<td>717 ± 139</td>
</tr>
<tr>
<td>Low IgG Anti-PGL-1 Responder (N=19)</td>
<td>0.212 ± 0.038</td>
<td>563 ± 41</td>
<td>417 ± 39</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values expressed as mean ± SEM

<sup>b</sup> t-test, one tailed
TABLE 2

Results obtained from thin layer chromatography (TLC) and ELISA analysis of the components extracted from armadillo plasma.

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Normal (Pre-Inf.)</th>
<th>Early</th>
<th>Mid</th>
<th>Late</th>
<th>Normal (Pre-Inf.)</th>
<th>Early</th>
<th>Mid</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>0.0</td>
<td>0.04</td>
<td>0.09</td>
<td>0.10</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>0.0</td>
<td>0.05</td>
<td>0.20</td>
<td>0.14</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.0</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>0.0</td>
<td>0.00</td>
<td>0.07</td>
<td>0.19</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>0.0</td>
<td>0.12</td>
<td>0.17</td>
<td>0.14</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>0.0</td>
<td>0.02</td>
<td>0.06</td>
<td>0.14</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>0.0</td>
<td>ND</td>
<td>0.01</td>
<td>0.08</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>0.0</td>
<td>0.00</td>
<td>0.00</td>
<td>0.10</td>
</tr>
</tbody>
</table>

a TLC values were scored subjectively based on intensity of the stained band.

b ELISA absorbance values for normal plasma were considered as baseline, and is presented here as zero. Subsequent values were derived by their subtraction from the preceding normal plasma value.

c ND = not done.
DISCUSSION

In the present study, the evolution of IgG and IgM antibodies of armadillos to PGL-I and to sonicated M. leprae were analyzed using ELISA and immunoblotting.

Longitudinal IgM and IgG responses of a larger group of armadillos to PGL-I were performed in order to confirm our previous findings (22) and to distinguish the high and low IgG anti-PGL-I responders for their further characterization regarding their antibody response to other M. leprae components.

The results of the present study substantiate our previous findings (22), in that, IgM anti-PGL-I absorbance values increase with time post-inoculation. This response was quite homogeneous and it persists throughout the course of infection, probably due to the availability of a continuous source of antigen. In comparison to the IgM response, the absorbance values for IgG anti-PGL-I were considerably higher and the response was heterogeneous. As reported previously (22), and also in this study, due to the heterogeneity in absorbance values for IgG anti-PGL-I, we were able to separate armadillos into two groups. Group A represents those with high absorbance values for IgG anti-PGL-I response, whereas Group B represents the armadillos with low absorbance values for IgG anti-PGL-I. The animals in Group A, in relation to those in Group B, were capable of delaying the dissemination of M. leprae infection as measured by time to appearance of AFB in ear biopsies. These animals also had a longer life span. These differences existing among the two groups were statistically significant when a larger group of animals was analyzed.
In human studies, Levis, et al. (17) have reported high IgG anti-PGL-I in some patients with tuberculous (BT) leprosy, a form of leprosy that is associated with controlling the infection. When Gormus, et al. (3) analyzed longitudinal serum samples of M. leprae-infected sooty mangabey monkeys for antibodies of IgG and IgM class to PGL-I, they reported that high IgG and low IgM anti-PGL-I levels are associated with less severe disease. Therefore, there may be an association between elevated IgG anti-PGL-I responses and upgrading of immunological responsiveness to M. leprae.

We had previously observed a sharp drop in absorbance values for IgG anti-PGL-I among 4 of 11 animals during the latter phase of infection and at a time when the animals were moribund (22). Significant decrease in IgG anti-PGL-I levels have also been described in M. leprae-inoculated sooty mangabey monkeys (8). These usually preceded and/or corresponded to periods of clinical progression of the leprosy symptoms. Since the drop in absorbance values was in parallel to the systemic dissemination of infection as manifested by bacteria in buffy coats, it was speculated that high concentrations of antigens like PGL-I in the plasma could influence the results of the antibody detection assay by in vivo complexing of antigens with some or all of the serum antibodies. Previous investigators have demonstrated that the 3,6-di-o-methyl-β-D-glucopyranose is the hapten determinant of the species-specific glycolipids (4,7,11). Therefore, due to the possible monovalent nature of PGL-I upon antigen antibody interaction one would not expect the formation of a lattice as commonly seen in precipitating immune complexes. This may explain our difficulty in
demonstrating immune complexes using polyethylene glycol (data not shown). Consequently, we analyzed the plasma samples for the presence of PGL-I. Analysis of selected plasma samples of 8 representative animals, 3 from Group A and 5 from Group B, indicated the presence of PGL-I in the plasma of 6 out of 8 animals. This was based on the migration pattern of the extracted component on TLC as compared with that of standard PGL-I and confirmed by its immunoreactivity with MAB F8b4 using ELISA. The presence of PGL-I in plasma was observed primarily in samples collected during the terminal phase of disease and correspondingly at a time in which a drop in IgG anti-PGL-I absorbance value was observed. Therefore, it is believed that this drop in absorbance value may be due to formation of complexes between high affinity IgG molecules and PGL-I in the plasma. This may be viewed as in vivo antigen excess in which the degree of drop in absorbance value may vary based on the amount of antigen bound to antibody prior to the application of plasma to the antibody detection assay. A significant drop in absorbance values for IgM anti-PGL-I antibodies was not readily observed among animals in Group A and B. A possible explanation could be due to a higher concentration of high affinity IgG anti-PGL-I molecules which out compete the low affinity IgM anti-PGL-I molecules.

Finally, the ability to detect \textit{M. leprae} antigens like PGL-I in biological samples such as plasma may provide a potentially useful tool for the diagnosis of lepromatous leprosy, but such antigens do not appear promissory for early detection of disease in armadillos because they are usually abundant at a time in which clinical signs and symptoms are about to take place. Presence of PGL-I in plasma of
human patients was also reported (5,23) and its significance in
detection of subclinical leprosy has yet to be determined.

The plasma of selected animals in Group A and B were further
analyzed for antibodies of IgM and IgG class to sonicated M. leprae
components by means of immunoblotting. The predominant IgM and IgG
responses were directed toward the M. leprae components with
molecular weights ranging from 21 - 45-kDa. Similar observations,
particularly with respect to the material with migration pattern at
the region of 33-kDa, was also reported in lepromatous patients (2).
The sera from armadillos having systemic infection with M. leprae
were also shown to react significantly with 33-kDa component (2,21).
We have previously shown that the components with broad diffuse
staining bands are glycoprotein in nature (21). Others have also
shown, upon electrophoresis and immunoblotting, that soluble
fractions of disrupted M. leprae produced a major antigen with an
apparent molecular mass of 30 - 50-kDa (1,13,18). This product has
now been identified as LAM (12). Based on our observations and those
of others, it appears that the major armadillo antibody activities
are directed against LAM. This could reflect the relative
immuno-dominance and/or accessibility of LAM in intact bacilli.
Furthermore, the detection of antibodies to LAM in the plasma of
armadillos prior to their inoculation with M. leprae is suggestive of
the ubiquitous nature of the antigen.

It is usually difficult to verify whether the 33-kDa
glycoprotein is a single substance or a mixture of 2 or 3 substances
with similar molecular weights. Based on the IgG immunoblot results
seen in Figure 4, it appears that beneath this common broad diffuse
band of immunoreactivity at the region of 33-kDa, there are as many as 3 distinct bands of immunoreactivity. As usually seen in IgM-type response (Figure 4), these components were not readily observed. A typical example of one such molecule is the 28-kDa protein. Recently, the gene for a 28-kDa protein of M. leprae has been cloned and was shown to be an important target of the humoral response in lepromatous leprosy (3). Further analysis of these components may prove important in the immunology of leprosy.

The 65-kDa protein was also recognized as antibody evoking immunogen in armadillos. Although in our previous study (21), we have shown the ability of this molecule in inducing antibody of IgG class, most animals analyzed here had antibody of IgM class to this molecule. However, the one animal with antibody activity to multiple components of M. leprae also had a very intense band of IgG immunoreactivity to the 65-kDa protein. These observations suggest the individual animal variation in antibody response to the 65-kDa molecule. Also, antibody responses of armadillos to the IIIE9 epitope of the 65-kDa protein of M. leprae have been analyzed using a competition binding assay. This assay incorporated crude cell wall extract of M. leprae or purified recombinant 65-kDa as antigen source. This epitope did not appear to be immunogenic. (Manuscript submitted). Finally, attempts were made to define lymphocyte blast transformation differences in high and low IgG PGL-I responders. However, the results were highly variable within and between groups (data not shown). In conclusion, as shown here, armadillo antibody activity increased significantly with time post-inoculation and this was directed primarily toward LAM as well as PGL-I. Furthermore, all
animals, regardless of their ability in mounting an elevated level of IgG anti-PGL-I response, showed a similar longitudinal pattern of IgG and IgM response to sonicated M. leprae components.
ACKNOWLEDGMENTS

This work was supported in part by grants from the American Leprosy Missions and the Hansen's Disease Foundation. The authors wish to express their appreciation to personnel of the Microbiology Research Department for making available the armadillo plasma samples. We are also grateful to Mr. Melvyn Morales for the computer analysis, Mr. Sterling Christy for processing and storing the plasma samples, and to Ms. Rosemary Hauge for secretarial assistance.
REFERENCES


Chapter IV  Confirmation of a false positive result associated with a competition inhibition assay used for detecting antibodies to a protein epitope of *Mycobacterium leprae*.

Submitted for publication to the *Journal of Clinical & Experimental Immunology*
ABSTRACT

A competitive antibody binding assay (CABA) was developed to detect antibodies in infected armadillos and leprosy patients which compete with an M. leprae-specific 125I monoclonal antibody IIIE9 (MAB-IIIE9) for the species-specific M. leprae-IIIE9 epitope on the 65-kDa protein. The CABA results suggest armadillos and leprosy patients produce antibodies that inhibit the binding of 125I-IIIE9 MAB to the IIIE9 epitope on crude, native 65-kDa protein preparations. When purified, recombinant 65-kDa protein was substituted for crude antigen, there was no evidence in the CABA of antibody to the IIIE9 epitope. False-positive results, possibly induced by steric hindrance, are likely to be associated with CABA which incorporate crude cell wall extracts as solid phase antigen.
INTRODUCTION

Activation of the immune system during infection generally leads to the proliferation and clonal expansion of specific T and B lymphocytes producing among other things, lymphokines capable of modulating the actions of other lymphocytes and accessory cells, as well as specific antibodies directed against antigenic determinants of the infectious organism. Serodiagnostic tests have been designed to take advantage of the presence of species-specific antibodies produced during the host's immune response and have provided powerful tools to diagnose and monitor infectious diseases.

Development of serological tests designed to detect a specific antibody population requires either the purified target antigen, in a form capable of binding population antibody present in test samples, or a second antibody preparation directed against species-specific antigenic determinants which can be used in a competitive antibody binding assay (CABA). The CABA is particularly suited when purified antigen preparations from the infectious agent are not available as is the case with difficult-to-grow microorganisms, such as M. leprae. The CABA has been employed to detect antibodies to specific protein antigenic determinants of M. leprae and M. tuberculosis (16). In each of these assays monoclonal antibodies (MAB) were used as the competitive reagent and crude extracts of the mycobacteria were used as antigen.

The premise upon which mycobacterial CABA's are currently founded is that labeled MAB directed against a defined epitope in crude antigen extracts would be blocked by antibody in serum of humans exposed to this infectious agent. A major advantage of this assay is
that antibody, specific for a single epitope in serum which contains multiple specificity can be detected which mirrors that of the MAB. An additional advantage is that specificity is not lost by utilizing crude antigen extracts.

Conversely, a disadvantage of CABA which uses crude antigen preparation is the effect upon specific antibody binding (either labeled MAB or test sera) by other serum antibodies which bind to antigenic determinants located in close proximity to the target epitope. This phenomenon, referred to as steric hindrance, may play a role in blocking the specific reaction which may lead to false-positive results. One approach by which to examine the role of steric hindrance and the degree to which it influences a given set of data is to compare results derived from CABA in which crude antigen was employed with findings in experiments where purified native protein of recombinant protein has been used as the antigen source. Unfortunately, in most situations, purified native protein or recombinant protein containing the epitope of interest are not readily available. As a result most investigators continue to use the CABA with crude antigen.

We were interested in evaluating the potential of armadillo antibodies directed to an M. leprae-specific epitope (IIIE9) on the 65-kilodalton (kDa) protein, for development of an assay which detects subclinical leprosy. To circumvent the laborious antigen purification process and, taking advantage of available MAB to the IIIE9 epitope, we selected the competition antibody binding assay to analyze 175 serum samples for antibody to IIIE9 epitope collected from 26 animals during the course of M. leprae infection. During the course of this
study, recombinant 65-kDa protein (r65-kDa) became available and the specificity of our data was evaluated by comparison of results obtained from CABA developed with non-purified native antigen to that of CABA developed using purified r65-kDa protein. The findings suggest that false positive results, due to steric hindrance, can occur when crude cell wall extracts of M. leprae as the source of IIIE9 epitope, are used in the CABA.
MATERIALS AND METHODS

Armadillo sera

Serum samples were collected from 26 nine-banded armadillos prior to infection (day 0) and then at 3-month intervals for a period of one to three years. The conditions for adapting animals into the armadillo (Dasypus novemcinctus) colony and their maintenance have been described previously (17).

Patient sera

Serum samples taken from 4 leprosy patients were supplied by Dr. W. R. Levis (Bayley Seton Hospital Medical College, New York, NY). Each serum sample had been identified as positive for antibody to IIIE9 epitope in CABA using crude cell wall extract (personal communication).

Preparation of M. leprae extracts

Irradiated, lyophilized, M. leprae purified from lymph nodes of infected armadillos was provided by Dr. Patrick Brennan (NIH contract AI-52582, Colorado State University). Bacilli (24 mg dry weight) were suspended in 3 ml of 0.01 M phosphate buffered saline (PBS), pH 7.2. The organisms were disrupted by sonication for 30 min at 4°C at 130 watts on a sonifer cell disruber with a temperature control module (Model W1851, Heat System; Ultrasonics, Inc., North Tonawanda, N.Y., USA). The sonicated material was sedimented at 10,000 x g for 10 min at 4°C. The supernatant fluid (10 ks) had a protein concentration of 7.15 mg/ml using protein estimation assay (13). The pellet (10 kp) was resuspended in 3 ml of 0.01 M PBS, pH 7.2, and was determined to have 8 mg protein/ml. Aliquots of the 10 kp were frozen at -20°C.
The expression 10 kp and crude cell wall extract of *M. leprae* will be used interchangeably.

**Preparation of recombinant 65-kDa (r65-kDa) protein of *M. leprae***

The procedure used to clone and express the gene which encodes the 65-kDa protein has been described (12). Crude protein extract prepared from recombinant *E. coli* T65-3.61, expressing the 65-kDa protein, were prepared by disrupting the bacterial suspension in a French Press. A soluble form of the r65-kDa protein was prepared by resuspending the insoluble fraction of the bacterial lysate in PBS which contained 1% sodium dodecyl sulfate (SDS), 1mM EDTA and 1mM benzamidine HCl, [pH 7.0] following centrifugation at 15,000 x g x 15 min. The antigen preparation was equilibrated by dialysis against 50 mM tris-HCl, 150 mM NaCl, 10 mM EDTA, pH 8.0. Purification of r65-kDa, from the crude soluble extract, was accomplished using affinity chromatography with monoclonal antibody IIC8 (8).

**Immunoblot***

The immunoblot technique used to detect armadillo IgG antibodies to antigens of *M. leprae* was done as described previously (17). Briefly, 800 ul which contained 0.66 mg/ml of purified r65-kDa protein was mixed with 800 ul of 2 x buffer (0.1 M Tris-HCl, 4% SDS, 10% 2-mercapto ethanol (2ME), 01% glycerol, 0.025% bromophenol blue) boiled for 3 min and loaded (100 ul/well) onto a 12% polyacrylamide gel. The antigen was electrophoresed for 4 hr at 45 mA and then transferred to BA85 nitrocellulose paper (NCP) (Schleicher and Schuell, Inc., Keene, N.H., USA) at constant voltage (10v) overnight in a tris-glycine-methanol buffer, pH 8.0. The NCP was cut into
strips, and each strip was incubated with a 1:30 dilution of the appropriate armadillo serum for one hr at room temperature (RT). Following three wash steps in 1% bovine serum albumin (BSA)-PBS, the strips were incubated with rabbit anti-armadillo IgG, gamma-chain specific at 1:100 dilution for one hr. After washing in BSA-PBS, the strips were placed in a 1:500 dilution of goat anti-rabbit IgG, IgM, IgA-peroxidase conjugate for 1 hr, followed by the addition of H$_2$O$_2$/horseradish peroxidase (HRP) (BioRad, Richmond, Ca., USA) for 10 min at RT.

**Radiolabeling of monoclonal antibody (MAB) IIIE9**

Murine MAB IIIE9 was purified from ascites by MAPS II affinity chromatography by methods described by the manufacturer (BioRad, Richmond, Ca., USA), and iodinated with carrier-free Na$^{125}$I (ICN Pharmaceuticals, Inc., Irvine, Ca.) by the chloramine T method (9).

**Competition antibody binding assay (CABA)**

Immulon® I remove-a-well microtiter plates (Dynatech Laboratories, Va., USA) were coated with 100 ul of *M. leprae* 10 kp (0.2 mg/ml) or with 100 ul of affinity purified r65-kDa protein at 0.2 ug/ml dissolved in 60 mM carbonate buffer pH 9.2 as coating buffer. The assay was performed on these plates after one wash with 0.01 M PBS, pH 7.0 and a 30 min blocking step with 0.01 M PBS which contained 3% BSA. Briefly, 50 ul of iodinated MAB IIIE9 at 1:50 dilution containing 2 x 10$^6$ CPM and 50 ul of armadillo serum diluted 1:15 or 1:5 human serum were added to wells and incubated at 4°C overnight. Each well was washed 6 times with 0.01 M PBS pH 7.0 (250 ul per well) to remove unbound components and then flicked dried. Individual wells
were cut out from each plate and counted in a gamma counter (Model 4000, Beckman Instruments, Inc., Fullerton, Ca.) and the results were expressed as percent inhibition (%I) using the following formula:

\[
\%\text{ inhibition} = 100 - \%\text{ Bound}
\]

\[
\%\text{ Bound} = \left[ 1 - \left( \frac{\text{*CPM 100\% Bound - CPM test sample}}{\text{*cpm 100\% bound}} \right) \right] \times 100
\]

The 100% bound control was obtained from counts observed when \(^{125}\text{I-IIIE9}\) was added to antigen plates in the presence of 1% BSA/PBS. *CPM 100% bound = antigen + \(^{125}\text{I-IIIE9}\) MAB. All experimental values were corrected for background due to nonspecific binding of reagents with uncoated polystyrene remove-a-well plates. Background counts did not exceed 500 CPM.
RESULTS

Antibody competition analysis using *M. leprae* 10 kp

A titration of $^{125}$I-IIIE9 MAB against crude cell wall antigen extract of *M. leprae* (10kp), as the source of IIIE9 epitope, was done. In this experiment $^{125}$I-IIIE9 MAB, at $1 \times 10^6$ CPM, and 10 kp at 0.2 mg/ml, was chosen in order to maximize the sensitivity with minimal reagent usage (data not shown). The specificity of the CABA was examined in a competition between labeled IIIE9 MAB and non-labeled IIIE9 MAB. An irrelevant MAB D$_2$D$_3$ which recognizes a 28 kDa protein of *M. leprae* was included as a control. Binding of $^{125}$I-IIIE9 MAB to the IIIE9 epitope in the crude cell wall extract increased as the concentration of cold IIIE9 MAB was reduced (Fig. 1). In addition, irrelevant MAB D$_2$D$_3$ did not interfere with the binding of $^{125}$I-IIIE9 MAB to the IIIE9 epitope.

Normal armadillo sera as well as sera collected at 100 and 363 days post-inoculation were titrated against *M. leprae* (Fig. 2a and 2b). Based upon titration findings of sera collected at 363 days post-inoculation, a 1:30 dilution was chosen for the CABA. When the optimal conditions were determined, the assay was performed for detection of armadillo antibodies to the *M. leprae*-specific epitope (IIIE9) on the 65-kDa protein (Fig. 3). One-hundred seventy-five serum samples taken from 26 armadillos were examined. The results are presented as % inhibition. To determine a positive response a mean of the % inhibition by the normal sera (0 day) + 2 standard deviation was designated as baseline. Therefore, a positive response was defined as
bodies to the IIIE9 epitope, primarily during the time when animals are burdened with disseminated bacilli (Fig. 3). The level of M. leprae dissemination was assessed on the presence of acid-fast bacilli in the buffy coat or in ear snip biopsies.

IgG response of M. leprae-infected armadillos to r65-kDa protein

To determine if armadillo antibodies directed to the native 65-kDa protein also bind to r65-kDa, serum samples from 16 armadillos were tested by Western blot using purified r65-kDa as the antigen source. The serum samples tested were collected late in infection, after dissemination of M. leprae had occurred. Immunoreactivity was observed at the region of 65-kDa in the sera of 15 of the 16 animals tested (Fig. 4). Pre-infection serum showed no evidence of anti-r65-kDa activity.

Antibody competition analysis using affinity purified r65-kDa protein

A preliminary titration indicated that 125I-III E9 MAB (1 x 10^6 CPM) and purified r65-kDa at 0.2 ug/ml was optimal for the assay (data not shown). When the specificity of the CABA, purified r65-kDa protein as the source of IIIE9 epitope was examined (Fig. 5), the binding characteristics were similar to those seen for native IIIE9 epitope (Fig. 2). Next, the CABA incorporating r65-kDa protein was performed to assess the presence of the armadillo antibodies produced to the murine MAB defined, M. leprae-specific epitope (III E9). Pre-infection serum and post-infection 270 and 360 days into M. leprae infection (or beyond the stated dates) were tested in this experiment because these sera produced positive response at 360 days (or beyond) in the assay in which crude cell wall extract was used as the source.
of 65-kDa protein. In this experiment the results are presented as %
inhibition and a positive response was defined as ≥ 18% inhibition
(Fig. 6). Only 4 animals were positive for anti-IIIE9 activity. One
animal showed low level of activity in the pre-infection serum only.

Analysis of leprosy patients sera for antibody to IIIE9 epitope in

CABA

A preliminary titration of patients sera in the CABA in which
crude cell wall extract was the source of IIIE9 epitope revealed that
a serum dilution of 1:10 was optimal. Four serum samples, from
different patients, were then tested by CABA against crude cell wall
extract (at 0.2 mg/ml) or purified r65-kDa protein (at 0.2 mg/ml).
The results from CABA using crude cell wall extract were consistent
with the findings reported by Dr. Levis and associates (personal
communication). When the same serum samples were analyzed in CABA
using purified r65-kDa, no inhibition of binding of 125I-IIIE9 MAB to
the IIIE9 epitope (Table 1) was observed.
Figure 1. Examination of the specificity for CABA using 10 kp. Crude cell wall extract of *M. leprae* (10 kp) was diluted to contain 0.2 mg/ml in 0.05 M carbonate-bicarbonate, pH 9.2 coating buffer. One-hundred ul of the cell wall extract was added to each well of the microtiter plate. The effect of relevant (cold IIIE9) and irrelevant (cold D2D3) MAB's on binding of $^{125}$I-IIIE9 MAB to the IIIE9 epitope was analyzed. Radiolabeled MAB was used at a final dilution to contain $1 \times 10^6$ CPM along with a serial dilution of cold MAB's ranging from $10^{-1}$ to $10^{-7}$. 
Figure 2a. Titration of armadillo sera collected prior to the experimental inoculation with M. leprae (normal sera). Technical procedure for coating microtiter plates is same as Fig. 1. Armadillo sera at dilutions ranging from $10^{-1}$ to $10^{-3}$ were used. $^{125}$I-IIIE9 was used at final dilution containing $1 \times 10^6$ CPM.
A = normal sera, n of 6.
B = non-specific binding (no antigen).
LOG DILUTION OF THE SERA

125I-IIIE9 MAB cpm x 10^3

-3 -2 -1
Figure 2b. Titration of armadillo sera collected at 100 and 363 days post-inoculation with M. leprae. Technical procedure is same as Fig. 2a.

A = 100 days post-inoculation.
B = 363 days post-inoculation.
C = non-specific binding (no antigen).
LOG DILUTION OF THE SERA

125I-IIIE9 MAB CPM x 10^3

A

B

C

0

-3

-2

-1

LOG DILUTION OF THE SERA
Figure 3. Detection of armadillo antibodies directed to the M. leprae-specific epitope (IIIE9) of 65-kDa protein prior to and during the course of experimental infection. Technical procedure for coating microtiter plates is same as Fig. 1. Armadillo sera at final dilution of 1:30 and 125I-IIIE9 at final dilution containing 1 x 10^6 CPM were used. For determining a positive response a mean of the % inhibition of the normal sera (0 day) + 2 S.D. was designated as baseline. A positive response was defined as > 22% inhibition.
MEAN of normal sera (0 day) + 2 S.D.
Figure 4. Immunoblot to demonstrate the antibody binding of M. leprae-infected armadillos to the r65-kDa protein of M. leprae. Purified r65-kDa protein (0.658 mg/ml) was analyzed by immunoblotting. Nitrocellulose paper (NCP) was incubated with sera from normal or M. leprae-infected armadillos at 1:50 dilution, washed, and incubated with rabbit anti-armadillo gamma-chain at 1:1000 dilution. The NCP's were then washed, and incubated with peroxidase conjugated goat anti-rabbit IgG, M, A at 1:500 dilution. The NCP was washed and treated with dye-developing reagent (H₂O₂, 4-chloro-1-naphthol).

Lane A = low molecular weight protein standards: phosphorylase b (92-kDa), BSA (66-kDa), ovalbumin (45-kDa), carbonic anhydrase (31-kDa), soybean trypsin inhibitor (21-kDa), lysozyme (14-kDa); Lane B and every other alternate lane = normal serum; Lane C and every other alternate lane = serum from infected armadillos.
Figure 5. Examination of the specificity for CABA using r65-kDa protein. Purified r65-kDa protein of *M. leprae* was diluted to contain 0.2 ug/ml in 0.05 M carbonate-bicarbonate, pH 9.2 coating buffer. One-hundred ml of the diluted r65-kDa was added to each well of the microtiter plate. The effect of relevant (cold IIIE9) and irrelevant (cold DpD3, cold IIIC8) MAB's on binding of 125I-IIIE9 MAB to the IIIE9 epitope was analyzed. Radiolabeled MAB was used at a final dilution to contain 1 x 10⁶ CPM along with a serial dilution of cold MAB's ranging from 10⁻¹ to 10⁻⁷.
125I-IIIE9 M R B CPM X 10^3

LOG DILUTION OF COLD MAB

- IIE9 anti-65 Kd
- D2D3 anti-28 Kd
- IIC8 anti-65 Kd
Figure 6. CABA which incorporate purified r65-kDa protein as the source of IIIE9 epitope. Technical procedure for coating microtiter plates is same as Fig. 5. Armadillo sera at final dilution of 1:30 and 125I-IIIE9 at final dilution containing 1 x 10^6 CPM were used. For determining a positive response a mean of the % inhibition of the normal sera (0 day) + 2 S.D. was designated as baseline. A positive response was defined as > 10% inhibition.
INHIBITION

MEAN of normal sera (0 day) + 2 S.D.

% INHIBITION

CLASS INTERVALS (DAYS)
TABLE 1

Comparison of a competitive antibody binding assay (CABA) which incorporated crude cell wall extract or purified recombinant 65kDa protein as the source of the IIIE9 epitope

<table>
<thead>
<tr>
<th>Serum from Leprosy Patients</th>
<th>5 Inhibition using crude Cell wall extract</th>
<th>% Inhibition Using purified Recombinant 65-kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>99.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>A 43 49</td>
<td>B 0</td>
</tr>
<tr>
<td>98.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24 27</td>
<td>0</td>
</tr>
<tr>
<td>67&lt;sup&gt;c&lt;/sup&gt;</td>
<td>31 51</td>
<td>0</td>
</tr>
<tr>
<td>72&lt;sup&gt;d&lt;/sup&gt;</td>
<td>54 58</td>
<td>0</td>
</tr>
</tbody>
</table>

A = Inhibition as determined in the laboratory of Dr. W. Levis.
B = Inhibition as determined in the laboratory at the Gillis W. Long Hansen's Disease Center.
DISCUSSION

Many of the antigenic determinants of mycobacteria are expressed by heterologous mycobacterial species; and as a result, antibodies to one mycobacterium species will often react with another species. Recently, protein antigens of M. lepra have been the focus of intensive study. Using MABs, it has been shown that certain protein epitopes of M. lepra are species-specific (6). A cell-wall associated 65-kDa protein molecule is an immunodominant component of M. lepra, M. tuberculosis, and BCG. The 65-kDa molecule has one M. lepra species-specific epitope (IIIE9) and a minimum of 13 epitopes expressed by heterologous species (1). The 65-kDa protein has been shown to be immunogenic in both humans and mice. Antibody, as well as T-cell clones reactive to this molecule, have been isolated from leprosy patients, their contacts, tuberculosis patients, and individuals responsive to purified protein derivative (4, 5, 15). These findings have encouraged investigators to focus on this molecule as the target antigen for development of a serodiagnostic assay. The method used to detect antibody directed to a given epitope, located within a molecule, is competitive antibody binding assay (3, 7, 14). Levis et al. (12), isolated a crude cell wall extract of M. lepra as antigen in the CABA to assess leprosy patient serum for activity against the 65-kDa IIIE9 epitope in the presence of 125I MAB. They reported that 19/59 multibacillary patients had antibody directing to the M. lepra-specific epitope (IIIE9) on 65-kDa protein, while no antibody to this epitope was detected in the serum of 15 paucibacillary patients.
Since armadillos serve as the animal model for multibacillary leprosy (11, we tested armadillo sera for the presence of detectable antibody to the IIIE9 epitope. The CABA which utilized 10 kp, resulted in 50% inhibition in binding the 125I-IIIE9 MAB to the IIIE9 epitope, observed generally with serum collected 360-540 days post-infection. These findings suggest that the IIIE9 epitope is immunogenic in armadillos. Conversely, when purified r65-kDa protein was used in the CABA, in place of crude antigen preparations, no antibody activity to this epitope was detected. Immunoblot analysis of armadillo sera to r65-kDa protein, on the other hand, showed that the armadillo produces antibody [IgG class] to the 65-kDa protein. It appears armadillos produce antibody to the 65-kDa protein, which is not directed toward or reactive with the IIIE9 epitope. Consequently, the lack of concordance between the two CABA may be due to steric hindrance caused by interaction of antibodies with heterologous specificity to epitopes other than IIIE9 present as solid phase antigen in the system.

To substantiate this finding, we retested leprosy patient sera (supplied by Dr. W.R. Levis) which was previously identified positive for activity to the IIIE9 epitope, when crude native antigen was used. In our laboratory these sera were also positive in CABA using crude cell wall extract, however, these sera samples were negative in the CABA using purified r65-kDa protein.

In a time course analysis of infected armadillo sera tested in an immunoblot assay using sonicated \textit{M. leprae} as antigen source, we have observed that these animals produce elevated levels of antibody titers to \textit{M. leprae} components with molecular weights which range from
21-45-kDa. The sera of some animals recognize serologically larger repertoires of *M. leprae* components in the later stages of post-inoculation with *M. leprae* (personal observation). Immunoblot studies done with sera of human leprosy patients revealed that lepromatous leprosy patients produce antibody to various *M. leprae* components (2). With these findings in mind it is believed that the lack of concordance between findings of CABA using crude cell wall extract versus those of CABA using purified r65-kDa protein is most likely the result of steric hindrance induced by irrelevant antibody molecules present in polyclonal serum.

The IIIE9 epitope of *M. leprae* does not appear to be immunogenic in the armadillo or the leprosy patient. Although serum from greater numbers of leprosy patients must be analyzed, the influence of steric hindrance must be considered when crude antigen preparations are employed especially with serum taken from patients with multibacillary leprosy, who produce antibody to a wide spectrum of components in the bacillus.
ACKNOWLEDGEMENTS

This work was supported in part by grants from the American Leprosy Missions and the Hansen's Disease Foundation. The authors wish to express their appreciation to personnel of the Microbiology Research Department for making available the armadillo plasma samples. We are grateful to Mr. Melvyn Morales for the computer analysis, Mr. Sterling Christy for processing and storing the plasma samples, and to Ms. Penne Cason for secretarial assistance. Our appreciation also goes to Dr. R.S. Siebling of the Department of Microbiology at Louisiana State University for the critical review of this manuscript as well as thoughtful discussions.
REFERENCES


The mechanism by which M. leprae eludes the host defense system to cause disease in man and armadillos is unknown. Since protection against disease is associated with an appropriate host immune response to M. leprae, delineation of the antigenic constitution of M. leprae is essential to the understanding of this process.

This study was undertaken to characterize the antigenic components of M. leprae which evoked IgG and/or IgM antibody responses in armadillos experimentally inoculated with M. leprae. When the longitudinal anti-PGL-I IgM and IgG responses of a group of eleven armadillos, which received the same inocula of M. leprae, and a large group of 30 armadillos, which received various inocula of M. leprae, was analyzed using ELISA, we observed that IgM anti-PGL-I absorbance values increased with time post-inoculation. This response was quite homogeneous and it persisted throughout the course of infection. The persistence of this response was probably due to the availability of a continuous source of antigen. This observation was in agreement with the findings reported by Truman, et al. (24).

In comparison to the absorbance values for the IgM response, the absorbance values for IgG anti-PGL-I were considerably higher and the response was heterogeneous. Due to the heterogeneity in absorbance values for IgG anti-PGL-I, we were able to separate armadillos into two groups. Group A represented those with high absorbance values for IgG anti-PGL-I response; whereas those in group B represented the armadillos with low absorbance values for IgG anti-PGL-I. The animals in group A, when compared to those in group B, were capable
of delaying the dissemination of \textit{M. lepraee} infection as measured by time to appearance of AFB in ear biopsies. These animals also had a longer life span.

In human studies, Levis, \textit{et al.} (17), have reported high IgG anti-PGL-I in some patients with tuberculoid (BT) leprosy, a form of leprosy that is associated with controlling the infection. When Gormus \textit{et al.} (9), analyzed longitudinal serum samples of \textit{M. lepraee}-infected Sooty mangabey monkeys for antibodies of IgG and IgM class to PGL-I, they reported that high IgG and low IgM anti-PGL-I levels are associated with less severe disease. Since \textit{M. lepraee} is an intracellular organism, antibodies to \textit{M. lepraee} are not thought to be protective in leprosy. Nevertheless, there may be an association between elevated IgG anti-PGL-I responses and upgrading of the immunological responsiveness to \textit{M. lepraee}.

We have also observed a sharp drop in absorbance values for IgG anti-PGL-I among some animals during the latter phase of infection and at a time when the animals were moribund. Significant decrease in IgG anti-PGL-I levels have also been described in \textit{M. lepraee}-inoculated Sooty mangabey monkeys (9). These usually preceded and/or corresponded to periods of clinical progression of the leprosy symptoms. Since the drop in absorbance values was in parallel to the systemic dissemination of infection as manifested by bacteria in buffy coats, it was speculated that high concentration of antigens like PGL-I in the plasma could influence the results of the antibody detection assay by \textit{in vivo} complexing of antigens with some or all of the serum antibodies. Previous investigators have demonstrated that the 3,6-di-o-methyl-B-D-glucopyranose is the hapten determinant of
the species-specific glycolipid (4, 7, 12). Therefore, due to the possible monovalent nature of PGL-I upon antigen antibody interaction one would not expect the formation of a lattice as commonly seen in precipitating immune complexes. Upon analysis of selected plasma samples, the presence of PGL-I in the plasma of 6 out of 8 animals became evident. This was based on the migration pattern of the extracted component on thin layer chromatography (TLC) as compared with that of standard PGL-I and confirmed by its immunoreactivity with monoclonal antibody (MAB) FSb4 using ELISA. The presence of PGL-I in plasma was observed primarily in samples collected during the terminal phase of disease and correspondingly at a time in which a drop in IgG anti-PGL-I absorbance value was observed. Therefore, it is believed that this drop in absorbance value may be due to formation of complexes between high affinity IgG molecules and PGL-I in the plasma. A significant drop in absorbance values for IgM anti-PGL-I antibodies was not readily observed among animals in group A and B. A possible explanation could be due to a larger concentration of high affinity IgG anti-PGL-I molecules which out-compete the low affinity IgM anti-PGL-I molecules. Furthermore, armadillo IgG and IgM antibody responses to various antigens of M. leprae were analyzed using isotypic-specific antisera by means of immunoblotting. Blots using supernatant fraction of sonicated M. leprae and developed for IgG antibodies to M. leprae showed multiple protein antigens (Mr=12-90-kDa) in some heavily-infected armadillos. In contrast, blots developed for IgM antibodies to M. leprae showed a single, broad, diffuse band of immunoreactivity at approximately 33-kDa. The 33-kDa immunogen was detectable with silver stain
modified for carbohydrate reactivity, suggesting the presence of a polysaccharide component. In addition, binding of $^{125}$I-concanavalin A to the 33-kDa component demonstrated the presence of mannose and/or glucose residues. Additionally, when we analyzed the IgG and IgM antibody responses of an infected armadillo to the highly cross-reactive mycobacterial antigen IAM (13) derived from M. tuberculosis, IgM activity was observed only to the broad diffuse bands at the regions of 28-30-kDa and 50-kDa. Comparison of the SDS-PAGE migration patterns and antibody binding characteristics of the 33-kDa material with that of the purified M. tuberculosis IAM (13) suggested that the two components were chemically and immunologically related. The ability of a 33-kDa molecule to elicit a strong antibody response in armadillos and humans infected with M. leprae has been reported by Chakrabarty, et al. (13). However, they reported no reactivity to 33-kDa in normal armadillos and uninfected humans by immunoblot analyses. These investigators used $^{125}$I-Staph-A protein, which detects primarily IgG antibodies. In our studies, the use of isotypic-specific antisera demonstrated the presence of IgG and primarily IgM antibodies to the 33-kDa antigen of M. leprae in sera of normal and infected animals. The immunoreactivity observed in sera from normal armadillos may indicate previous exposure of these armadillos to M. leprae or to a cross-reactive mycobacterial antigen present in the environment.

Next, longitudinal plasma samples of selected animals [animals representing those with high IgG anti-PGL-I response (group A) and those with low IgG anti-PGL-I response (group B)] were further analyzed for antibodies of IgM and IgG class to sonicated M. leprae
95

components by means of immunoblotting. The predominant IgM and IgG responses were directed toward the \textit{M. leprae} components with molecular weights ranging from 21-45-kDa. Comparison of immunoblot analyses of armadillo IgG responses to \textit{M. leprae} components with that of humans indicates that \textit{M. leprae}-infected armadillos generally recognize and produce antibodies to a larger number of \textit{M. leprae} components than multibacillary patients. For example, analysis of sera from \textit{M. leprae}-infected armadillos indicated that in addition to the immunoreactive components recognized by both armadillo and human sera, armadillo sera reacted to a group of \textit{M. leprae} components with molecular weights ranging from 45-kDa to 90-kDa and, among these, only the 86-kDa component of \textit{M. leprae} was recognized by human sera (16). Our results support earlier findings that armadillos produce antibody to a great diversity of \textit{M. leprae} antigens as opposed to the previously reported limited repertoire produced by leprosy patients. Differences in antibody profiles between experimentally \textit{M. leprae}-infected armadillos and humans afflicted with \textit{M. leprae} could be due to the effect of chemotherapy of patients as suggested by Klaster, \textit{et al.} (16), or as a result of immune recognition of various antigens governed by immune response genes.

In conclusion, relative to the comparison of immunoblot analysis of animals representing group A versus those representing group B, in both groups, we observed a similar longitudinal pattern of IgG and IgM response to sonicated \textit{M. leprae} components. Finally, the cell-wall associated 65-kDa protein of \textit{M. leprae}, a molecule which has been the focus of intensive study by many investigators (2, 19), was also recognized as antibody evoking immunogen in armadillos.
This molecule has been shown to be immunogenic in both humans and mice. Antibody, as well as T-cell clones reactive to this molecule, have been isolated from leprosy patients, their contacts, tuberculosis patients, and individuals responsive to purified protein derivative (6, 21). The 65-kDa molecule has one M. leprae species-specific epitope (IIIE9) and a minimum of 13 epitopes expressed by heterologous species (2). These findings have encouraged investigators, including us, to focus on this molecule as the potential target antigen for development of a serodiagnostic assay for detection of subclinical leprosy. The method generally used to detect antibody directed to a given epitope, located within a molecule, is competitive antibody binding assay (CABA) (5, 8, 20). The CABA is particularly suited when purified antigen preparations from the infectious agent are not available as is the case with difficult to grow microorganisms, such as M. leprae. In one such assay radiolabeled MAB is used as the competitive reagent and crude extracts of mycobacteria are used as antigen. The premise upon which mycobacterial CABA's are currently founded is that labeled MAB directed against a defined epitope in crude antigen extracts could be blocked by antibody in serum of humans exposed to this infectious agent.

Therefore, we analyzed 175 serum samples for antibody to IIIE9 epitope collected from 26 animals during the course of M. leprae infection, in a CABA which utilized crude cell wall extract of M. leprae as antigen source. The results indicated a 60% inhibition in binding of the 125I-IIIE9 to the IIIE9 epitope, generally in serum collected 360-540 days post-infection. These findings suggested that
the IIIE9 epitope is immunogenic in armadillos. In human studies, Levis, et al. (18) have assessed leprosy patient sera for activity against the 65-kDa IIIE9 epitope in CABA and reported that 19/59 multibacillary patients had antibody to the IIIE9 epitope.

A disadvantage of CABA which uses crude antigen preparation is the effect upon specific antibody binding (either labeled MAB or test sera) by other serum antibodies which bind to antigenic determinants located in close proximity to the target epitope. This phenomenon, referred to as steric hindrance, may play a role in blocking the specific reaction which may lead to false-positive results. One approach to examine the role of steric hindrance and the degree to which it influences a given set of data is to compare results derived from CABA in which crude antigen was employed with findings in experiments where purified native protein or recombinant protein has been used as the antigen source. During the course of this study, recombinant 65-kDa (r65-kDa) protein became available and the specificity of our data was evaluated in its comparison to the results obtained from CABA developed using purified r65-kDa protein. The results derived from one such comparison, using armadillo or patient sera, indicated that when purified r65-kDa protein was used in the CABA, in place of crude antigen preparations, no antibody activity to this epitope was detected.

It appears the lack of concordance between the two CABA may be due to steric hindrance caused by interaction of antibodies with heterologous specificity to epitopes other than IIIE9 present as solid phase antigen in the system.
In a time course analysis of infected armadillo sera tested in an immunoblot assay using sonicated *M. leprae* as antigen source, we have observed that these animals produce elevated levels of antibody titer to *M. leprae* components with molecular weights ranging from 21-45-kDa. The sera of some animals recognized serologically larger repertoires of *M. leprae* components in the later stages of post-inoculation with *M. leprae*. Immunoblot studies done with sera of human leprosy patients revealed that lepromatous leprosy patients produce antibody to various *M. leprae* components (3). With these findings in mind, it is believed that the lack of concordance between findings of CABA using crude cell well extract versus those of CABA using purified r65-kDa protein is most likely the result of steric hindrance induced by irrelevant antibody molecules present in polyclonal serum.
REFERENCES


VITA

Abdolreza Vadiee was born in Shiraz, Pars, Iran, on October 22, 1959. He was graduated from Rayvand High School in Shiraz, Pars, in May, 1978.

He continued his education at Southeastern Louisiana University and received a Bachelor of Science degree in Microbiology in May, 1983. He entered the Graduate School at Southeastern Louisiana University in 1983 and was awarded a Master of Science degree in Microbiology in 1985.

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Abdul Reza Vadiee

Major Field: Microbiology

Title of Dissertation: The Evolution of IgG and IgM Antibodies to Antigens of Mycobacterium leprae in Experimentally Inoculated Armadillos

Approved:

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EXAMINING COMMITTEE:

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Date of Examination:

September 8, 1989