1985

Host Responses to the Phenolic-Glycolipid-1 Antigen of Mycobacterium Leprae (Elisa, Leprosy, Epidemiology, Armadillo, Human).

Richard W. Truman

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HOST RESPONSES TO THE PHENOLIC-GLYCOLIPID-1 ANTIGEN OF MYCOBACTERIUM LEPRAE

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HOST RESPONSES TO THE
PHENOLIC-GLYCOLIPID-1 ANTIGEN
OF
MYCOBACTERIUM LEPRAE

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
Veterinary Medical Sciences
with option in
Veterinary Microbiology and Parasitology

by
Richard W. Truman
B.A. West Virginia University, 1974
M.S. Louisiana State University, 1978
May 1985
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For Jonah
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ABSTRACT

HOST RESPONSES TO THE PHENOLIC-GLYCOLIPID-1 ANTIGEN OF MYCOBACTERIUM LEPRAE

by

Richard W. Truman

Antibody responses to the apparently species specific phenolic-glycolipid-1 (Phen-Gl-1) antigen of *Mycobacterium leprae* were examined in humans and armadillos using enzyme-linked immunosorbent assays (ELISA). Statistical definitions for the interpretation of positive and negative reactions were derived. A retrospective serological survey of armadillos indicated that leprosy in the wild armadillo is a naturally acquired zoonosis. Presently 12.5% of the armadillos in 2 parishes in south central Louisiana have detectable IgM antibodies to Phen-Gl-1. Approximately 2.7% of these histologically exhibit clinical disease. Antibodies were not detected in Florida armadillo sera. Variations in prevalence rates were noted, and may be due to environmental conditions, population characteristics or some intricacies in the transmission of leprosy. Naturally acquired leprosy in the armadillo may be used as a model to study transmission and baseline data have been derived. The ELISA was shown to have application in the management of experimental leprosy infections in armadillos. Resistant armadillos were noted to have an irregular or
absent antibody response to the Phen-Gl-1 antigen over the course of an experimental infection. Armadillos infected in the wild also had an irregular IgM response. Susceptible armadillos appeared to have a long-term IgM antibody response to Phen-Gl-1 becoming detectable some 186 days post-experimental infection. This antibody remained detectable for up to 1140 days post-infection. Antibody responses of susceptible armadillos correlated with the harvestable load of *M. leprae* in liver tissues and ELISA absorbances successfully predicted a harvest result 97% of the time. IgM antibodies to Phen-Gl-1 were earlier and more reliable than other indicators of infection previously applied. IgM, IgA, and IgG antibodies to Phen-Gl-1 were detected in leprosy patients and contacts. IgM appeared to be the predominate isotype detectable. Human patients showed no significant correlation of antibody relative their clinical status. IgM antibodies to Phen-Gl-1 were depressed as a result of therapy with thalidomide. Monitoring Phen-Gl-1 antibodies in human patients is not predictive of patient status or reaction and does not seem indicated for clinical management.
CHAPTER 1: INTRODUCTION

Leprosy is a disease of certain antiquity yet infects approximately 11 million people in the world today (173). Caused by Mycobacterium leprae, the disease progresses slowly in man and can result in severe deformation, debilitation, and death. Patients present a complex spectrum of clinical and immunologic symptoms (27,85). They are plagued by a number of acute reactional states and management of the leprosy patient is complex. Early diagnosis of leprosy is helpful in avoiding pain and suffering, and techniques to aid diagnosis and management are greatly needed (58).

Serology holds hope for use in the early diagnosis of leprosy and may prove useful in the management of leprosy patients. However, the serology of M. leprae has been most complicated. Previous serologic techniques used undefined antigen mixtures and lacked both sensitivity and specificity (130). Recently a phenolic glycolipid antigen of M. leprae was isolated. The phenolic-glycolipid-1 (Phen-Gl-1) antigen is chemically defined and apparently species specific for M. leprae (22,33,78). The specificity of this antigen may alleviate many problems previously encountered in leprosy serology.

The purpose of these investigations was to provide basic information about the antibody response to the phenolic-glycolipid-1 antigen and to develop it further for
use in leprosy. The following chapters describe the
development, evaluation and application of enzyme-linked
immunosorbent assays (ELISA) for use in armadillos and
humans. The applications include epidemiology and
prospective management.
CHAPTER 2: REVIEW OF LITERATURE

Leprosy (Hansen's disease (HD); hanseniasis) is a chronic mycobacterial disease primarily affecting the peripheral nervous system and secondarily involving skin and other tissues. Generally a disease of the Third World, training in leprosy is usually neglected in developed countries \(^{(58)}\). To aid the reader's understanding, a brief review has been prepared. Characteristics of the \textit{M. leprae} organism, transmission, classification, epidemiology and immunology of leprosy are discussed in general. Emphasis has been given to serologic events in leprosy and the assay systems used to detect them.

\textbf{The organism:}

\textit{Mycobacterium leprae} was first described by Armauer Hansen in 1873 \(^{(59)}\). The organism is an acid fast bacillus of apparently high infectivity but low pathogenicity \(^{(84)}\). Although there are occasional reports to the contrary, a general consensus holds that \textit{M. leprae} has not been cultivated on artificial media and researchers must rely on \textit{in vivo} propagation methods as a source of \textit{M. leprae}. The mouse footpad, the nine banded armadillo \textit{(Dasypus novemcinctus)}, the mangabey monkey and a variety of immunodeficient animals have been shown to support growth of \textit{M. leprae}. Shepard first reported growth of \textit{M. leprae} (from nasal washings) in the mouse footpad in 1960.
(178). The mouse supports limited replication and the infection does not disseminate. This model is currently used for drug sensitivity evaluations, and evaluation of experimental vaccines. Growth of *M. lepra* in the armadillo was first reported by Kirchheimer and Storrs in 1971 (97). The armadillo uniquely sustains a disseminated infection and is in common use for the production of large quantities of *M. lepra*. Infection in the mangabey monkey has only recently been reported and, along with the armadillo, may develop as another animal model to study the host-parasite relationship in leprosy (117).

Identification of *M. lepra* is presumptively made on the basis of an acid-fast bacillus whose acid fastness is pyridine extractable (115) and has the ability to oxidize DOPA (144,145). More specific methods for identification, including antigenic analysis and DNA hybridization, are developing (8). Confirmation is usually dependent on a histological examination which requires presentation of a typical leprosy profile with acid-fast bacilli in peripheral nerves (86). *M. lepra* has no flagella or observable appendages, and appears to be nonmotile (143).

Antigenically, *M. lepra* are similar to organisms of the MAIS complex (*M. avium, M. intracellulare, M. scrofulaceum*) (12). They bear a number of antigens which cross react with these and other mycobacterial species. Using sera from lepromatous patients or antisera raised in rabbits, serological cross reactions have been noted with
M. avium, M. gallinarum, M. simiae, M. chitae, M. smegmatis, M. kansasii, M. phlei, M. fortuitum, M. balnei and M. bovis (BCG). Using lymphocyte blast transformation in humans, mice and rabbits sensitized with M. leprae: M. bovis (BCG), M. dudvallii, M. giluum, M. nonchromogenicum and M. vaccae were shown to cross react with M. leprae.

Skin test cross reactivities and monoclonal antibodies have added M. gordonae to the list. While the antigenic determinants responsible for cross reactions may not be major antigenic complexes of M. leprae, even minor complexes in mycobacteria can promote strong cross reactions. This phenomenon has been coined "Original Mycobacterial Sin". The cross reactivity of M. leprae with other mycobacterial species has complicated vaccination trials and attempts to monitor M. leprae-specific immune responses.

A number of investigators have sought to identify antigenic determinants that are specific for M. leprae. The first refinement of M. leprae antigen systems was the so-called antigen 7 by Harboe. This mixture of antigens was identified by gel diffusion studies with lepromatous sera on sonicated fractions of whole M. leprae. The majority of lepromatous patients examined had antibodies that precipitated this antigen. While recognized as a mixture of determinants, some sought to enhance the specificity of this antigen by absorbing test
sera with a variety of other mycobacterial species (e.g., BCG, M. avium, and M. nonchromogenicum) (61,67). Absorption to promote specificity did not meet the criteria for a species-specific antigen (61). Recently, Ivanyi and Gillis (50,81) independently reported a monoclonal antibody that reacts with a specific antigenic determinant on M. leprae. The epitope with which this antibody reacts is believed to be protein, but the determinant has not been defined (81,50).

Brennan (Colorado State University) has identified a specific antigen in the lipid fraction of M. leprae. A phenolic glycolipid in nature, its specificity is imparted in a trisaccharide terminus of: 3-0-Me-rhamnose; 2,3-d-0-Me-rhamnose and 3,6-d-0-Me-glucose glycosidically linked to the phenol substituent (21,77). The immunodominant portion of the molecule appears to be the terminal glucose residue (25). The lipid structure is related to mycoside-A of M. kansasii and is similar to other oligosaccharides found on mycobacteria of the MAIS complex (78). Serological cross reactivities have not been reported with other mycobacterial species. As yet, little is known of the immune response to this antigen.

Epidemiology:

The rate at which leprosy spreads depends on the proportion of susceptible persons in the population and their opportunity for contact with M. leprae. Adults are
thought to be relatively insusceptible; most attempts to infect volunteers have failed and conjugal leprosy is only about 5%. Children seem most susceptible. When there is leprosy in a family, up to 100% of the children may develop the disease (68). The incubation period for leprosy is thought to be 3 to 5 years. Shorter and longer periods of incubation have been reported. Although the bacilli do not cross the placenta, bacillary antigens do. The effect of these antigens on eventual susceptibility is uncertain.

Leprosy has world distribution, but is highest in tropical and sub-tropical climates. It is found less commonly in the Mediterranean population, Australian aborigines, and natives of Texas and Louisiana (58).

Transmission:

Transmission of leprosy may be direct or indirect, but direct contact may be more effective. Long-term close contact seems to be required for the establishment of infection. The exact site and mode of entry of *M. leprae* into the host has not been established. The organism probably enters the body as any other pathogen, either through the skin, the respiratory tract, or the gastrointestinal tract. Broken epithelium may be necessary for entry through the skin. Large numbers of organisms are shed in the nasal secretions of a leprosy patient with high bacillary load, which may imply a respiratory mode of transmission (84). The outcome of infection may be
determined by the organism's route of entry, the size of
the inoculum and the resulting host-parasite interaction.
The route of entry could determine how the organism is
presented to the immune system (193). The inoculum size to
which the potential host is exposed (either a single event
or chronically over time) could also influence the response
of the host immune system.

Environmental factors influencing transmission of
leprosy have been described. High relative humidity and
warm temperatures correlate with increased prevalence rates
of leprosy (80). Kazda recently isolated a M. leprae-like
organism from a sphagnum bog in Norway. The organism is
DOPA and pyridine positive, and reportedly bears the
phenolic glycolipid-1 antigen (92,25). The role of the
environment or environmental microorganisms as pathogens
or additional factors influencing the transmission of
leprosy has not been described. Stanford speculated that
environmental mycobacteria antigenically similar to
M. leprae may contribute to susceptibility for leprosy
(190). Antibodies elicited from previous exposure to
environmental mycobacteria may mask epitopes important in
the cell-mediated recognition of M. leprae (11).

The clinical form of disease presented by a given
individual may be dependent upon the route of entry or host
factors such as genetic makeup, nutrition and active
infections or therapy which may play a part in reduced host
resistance (84). In recent years, there has been evidence
that the immunological status of the host may be the most important factor. The clinical spectrum seen in leprosy is closely related to the status of the cell-mediated immunity (CMI) of the host. *M. leprae* are not eliminated from the body by humoral immunity (antibody) but are controlled or eliminated by the CMI (200).

**Classification:**

Disease classification schemes in leprosy must take into account the clinical spectrum of the disease, the bacterial load, the histopathological appearance of the lesions and/or the immunological status of the patient (85). One the most widely used classification systems is the five-group Ridley-Jopling classification scheme (153). This system classifies leprosy patients based on histopathologic features and the response to a lepromin test.

The lepromin test consists of intradermal injection of a phenol-treated suspension of heat-killed organisms harvested from tissue of a high bacillary load (102). Three types of responses have been described (149): The Medina reaction, which peaks at 4 to 6 hours, measures antibody; the Fernandez reaction detects the presence of competent T lymphocytes with a positive reaction at 48 to 72 hours as a delayed-type hypersensitivity reaction; the Mitsuda (or lepromin) reaction is noted grossly or histologically at the injection site 2 to 3 weeks post-
inoculation. A positive Mitsuda (well-defined tuberculoid granuloma) indicates that a person has the capacity to develop competent cell mediated immunity for *M. leprae*.

Across the Ridley-Jopling scale there are five classifications of leprosy, ranging from polar tuberculoid (TT) to polar lepromatous leprosy (LL). Tuberculoid leprosy is associated with a positive Fernandez (lepromin) reaction. Patients of this class have competent CMI but little circulating antibody is found (30). Clinically, there are few discrete asymmetrical lesions, typically large and erythematous or hypopigmented with a dry sometimes scaly surface. The outer border is sharply defined and elevated. The inner border is vague and slopes down to a flat, atropic center which exhibits anhidrosis anesthesia, and a lack of hair growth. Peripheral nerves in the region of the lesion may be thickened and palpable. Early manifestations of this form of leprosy may be sensory loss followed by motor loss possibly leading to deformities. Histologically, tuberculoid lesions are well-defined granulomas with a central area of mature epithelioid cells surrounded by a large lymphocytic infiltrate. The granuloma must extend to the epidermis. Langhans' giant cells may or may not be present and acid-fast stains seldom reveal organisms. Since the granulomas follow nerves, they are often elongated and organisms may be found in involved nerves (85,153).
On the opposite end of the spectrum is the patient with lepromatous leprosy. Lepromatous leprosy is characterized by negative Fernandez and negative Mitsuda reactions. These patients have a nonfunctional CMI for *M. leprae* and large quantities of antibody may be found. The hypergammaglobulinemia seen in these patients includes heightened antibody response to many antigens, some probably related to *M. leprae* and varying amounts of autoantibodies (30,113). Clinically, these patients have many diffuse and symmetrical lesions which may be macular, papular or nodular. Papules and nodules tend to be of normal skin color, but macules are usually hypopigmented and have vague indistinct edges with a shiny surface. Nerves are infected but not thickened in early lepromatous leprosy. Eventually there is hair loss and impaired sensation. Lesions may become infiltrated to distinct visibility and can progress to give characteristic leonine faces and madarosis seen in late lepromatous leprosy. Histologically, in lepromatous leprosy the epidermis is flattened and there is a clear zone separating the epidermis from the diffuse granulomatous infiltrate. The granuloma is composed primarily of macrophages with abundant vacuolated cytoplasm. These foamy macrophages are called lepra cells or Virchow cells. On acid-fast staining, many organisms are found inside these macrophages. Lymphocytes and plasma cells may be scattered in the
diffuse granuloma. Lymph nodes are often extensively involved (85,153).

Between the two extreme poles of leprosy, there are gradations of the disease with the midpoint designated as mid-borderline (BB) or dimorphous leprosy. Clinically, there are a number of lesions which are anesthetic at the center but have well-defined inner borders and less distinct outer edges. Histologically there is a mixed infiltrate showing both histiocytes and epithelioid cells as well as numerous lymphocytes (85,153).

Between borderline and tuberculoid, those borderline patients showing predominately tuberculoid features (but too disseminated to classify as TT) are termed borderline-tuberculoid (BT). The lesions are smaller and more numerous, and satellite lesions may be present. The edges of these lesions are less well defined than those in polar tuberculoid. Histologically, BT granulomas consist of epithelioid cells and lymphocytes forming a band in the dermis separated from the upper dermis by a clear area (85,153).

The borderline lepromatous (BL) patient shows more of the features of lepromatous leprosy. Their plaquish lesions have an inverted saucer appearance with a sloping outer margin, a steep inner margin, and patchy anesthesia over some of the lesions. The histology of a BL lesion is that of focal collections of inflammatory cells — macrophages, lymphocytes, and epithelioid cells. Acid-fast
staining will reveal many bacilli within the macrophage (85, 153).

Indeterminant (I) leprosy is considered the earliest clinical manifestation of the disease. There is usually a single macular lesion which is usually hypopigmented and rarely erythematous with indefinite edges. The histology of this lesion is nonspecific, having collections of mononuclear cells around skin adnexa, especially dermal nerves (85, 153).

Classification of leprosy is important for prognostic as well as treatment purposes. Classification is most accurate when based on histopathologic as well as clinical evaluation. In 1982, Job and Chacko modified the Ripley-Jopling classification scheme to include a purely neural form described earlier by Dharmendra and to designate the mid-borderline (BB) condition as so uncommon and unstable as to represent a crossover point rather than a group in the classification (85).

**Treatment:**

Present treatment recommendations for leprosy are a combined regimen of dapsone, rifampin and clofazimine in patients with multibacillary disease and dapsone plus rifampin in patients with few bacilli or lesions (179).
General leprosy immunology:

The lack of an antibody response in tuberculoid leprosy and the lack of a cell-mediated response in lepromatous leprosy has been termed split anergy. The split anergy of leprosy is an interesting immunological phenomenon and the mechanism of this anergy has been widely debated. Some have suggested that the defect of cell-mediated immunity in lepromatous leprosy is generalized or nonspecific (28,212), while most feel the impairment is specific to M. leprae (18,38,52,151,213).

Lepromatous patients are capable of mounting a high level of cellular resistance to other intracellular parasites (e.g., Leishmania braziliensis) (38), and an increased incidence of other infections and tumors in leprosy patients is not seen (200). Stoner has suggested that leakage of bacilli into circulation at an early stage of infection might preferentially stimulate the central lymphon compartment while circumventing the peripheral lymphon compartment. He suggested that there is a subsequent development of suppressor cells rather than normal activation of the effector cells essential to establish CMI (193).

Cell-mediated immunity relies on the interaction of macrophages, T lymphocytes, and possibly natural killer cells (207). The split anergy of leprosy may be the result of some dysfunction in the communication of these cells. M. leprae-induced impairment of the lymphocyte/macrophage
interaction has been reported in patients with lepromatous leprosy (55). Convit showed that macrophages of lepromatous patients could eliminate *M. leprae* when properly stimulated (39). Such proper stimulation required a population of sensitized lymphocytes. Absence of these lymphocytes, either functionally or physically, could account for the inability of lepromatous patients to respond adequately to *M. leprae* antigens (53).

Considering the granulomas of leprosy, rhinoscleroma, and sarcoidosis, Modlin used monoclonal antibody to demonstrate two different patterns (121,122). Granulomas of tuberculoid leprosy and sarcoidosis had T helper cells throughout the granuloma with T suppressor cells in the mantle of the lymphocytes surrounding the well-formed granuloma. Both helper and suppressor cells were diffusely scattered throughout the loose granulomas of lepromatous leprosy and rhinoscleroma. No surrounding mantle of T suppressor cells was present. It was postulated that the close association of T helpers and epithelioid macrophages in well-formed granulomas may reflect active cooperation to promote an effective immune response. The surrounding T suppressors may serve to contain the granulomatous reaction. The haphazard arrangement seen in the diffuse granulomas of lepromatous leprosy might reflect a relative inefficient host response that allows *M. leprae* proliferation. Van Voorhis has reported that T suppressor cells are scattered throughout the diffuse granulomas of lepromatous leprosy,
and that T helper cells were present in the well-organized granulomas of tuberculoid leprosy (206). T suppressor cells from lepromatous patients also failed to suppress normal B cell responses to the pokeweed mitogen, and B lymphocytes of lepromatous patients showed a higher mitogen response than normal controls or patients with tuberculoid leprosy (26,31).

While there is much evidence to indicate a T cell defect in lepromatous leprosy, splenic entrapment and inhibition of recirculation of lymphocytes might prevent their response to antigenic stimuli. Splenic entrapment of lymphocytes has been reported in the murine leprosy model (26). Two stages of splenic suppressor activity were also demonstrated in that model: early suppression was mediated by macrophages and late suppression was by T lymphocytes. Thus, the specificity and mechanism of the split anergy of leprosy is not well understood, but leprosy patients are markedly different from normal individuals and show great heterogeneity among themselves (26).

Reactions:

Leprosy patients also suffer a number of severe immunological reactions. An important focus of the immunology of leprosy is to develop methods that would aid in the management of these reactions. The reactional states of leprosy are broadly placed into two categories:
type I lepra or reversal reactions, and type II lepra or erythema nodosum leprosum.

Reversal reactions (type I lepra) occur in patients in the nonpolar and polar extremes of the leprosy spectrum. These reactions are brought about by a change in the CMI of a patient \(68,70,88\). They are by nature type IV hypersensitivity reactions according to the Gell and Coombs' scale \(41\). Clinically, patients exhibit increased erythema and edema in existing skin lesions and swelling of nerves \(37\). Histologically, there is a shift in classification; treated patients undergoing reversal, by definition move toward tuberculoid and have increased lymphocytes in their granulomas with a decrease in the number of viable (solid-staining) and total acid-fast bacilli \(68,70,88\).

Also grouped with the type I lepra reactions is the so-called "downgrading" reaction of the borderline leprosy patient \(68,70,88\). Clinical features here are similar to those for the reversal reaction and manifest as a delayed-type hypersensitivity \(158,159\). However, these patients are moving toward the lepromatous end of the spectrum.

The Akuter-Schub (acute panic) reaction is rare and occurs in the borderline lepromatous patient who is downgraded to lepromatous. With treatment, the patient has a slow upgrading until at some point the CMI is activated and the patient experiences multiple reversal reactions at sites where there is residual bacteria. This is a serious
reaction, and results in multiple skin lesions and nerve
involvement (68,70,88).

Drugs used in suppression of reversal reactions are
steroids and clofazimine (68,70).

Type II lepra reactions occur in the lepromatous
spectrum of leprosy. They are manifest in a rare event
known as Lucio phenomenon or in the more common reaction
erythema nodosum leprosum (ENL). Lucio occurs only rarely
and only in a specific sub-type of lepromatous leprosy,
predominately in patients in Sinalou, Mexico. It is a
severe complication of lepromatous leprosy characterized
clinically by crops of irregular, stellate infarctions of
the skin without nodular lesions (37). These infarctions
result in necrotic, ulcerated lesions which are sharply
separated from the surrounding non-inflamed skin, and
sloughing occurs. Lucio acts as a severe vasculitis of
deep dermal vessels with thrombosis effectively cutting off
blood supply leading to deep ulceration (68).

Erythema nodosum leprosum (ENL) or type II lepra
reaction is the most common complication of lepromatous
leprosy and accounts for the majority of permanent sequelae
in this disease type. Approximately 50% of lepromatous
leprosy patients will experience ENL some time during the
course of their disease. Although ENL usually occurs by
the end of the first year of sulfone therapy, it may also
manifest in the untreated patient. Therefore, ENL is
considered a manifestation of the disease rather than a
complication of therapy (150). ENL can be triggered by intercurrent infections, vaccinations, pregnancy and physical or emotional stress (17,68,70,88). Clinically, ENL is characterized by crops of tender erythematous papules or nodules on grossly uninvolved skin (37,68,70,88,149,200). The lesions are edematous and evanescent, lasting only 24 to 48 hours before spontaneous regression and may leave a faint bluish hyperpigmentation with slight desquamation of the surface (37,68,70,88). Eruption may be accompanied by general malaise, vague pains and an elevation in temperature. Lesions usually appear on the face, trunk, and extremities with the extensor surfaces of the limbs being favorite sites (68,70,87).

ENL exists in three phases (68,70,87): the acute phase (described above) is often accompanied by systemic symptoms of intermittent fever, polyarthralgia, polyneuritis, tender lymphadenopathy, iridocyclitis, and orchitis. This phase resolves in two to three weeks. In the subacute phase of ENL, crops of nodules recur at frequent intervals taking two to three months to resolve. In the chronic phase, nodules may continue to erupt with attacks lasting for several years. The patient with ENL will have an elevated sedimentation rate, a leukocytosis and proteinuria (37,212). Glomerulonephritis can be a complication of severe ENL. In severe episodes of ENL, lesions may become hemorrhagic, necrotic and ulcerated (68,70,87).
ENL is viewed as a hypersensitivity reaction and inflammation in the episode is considered of an allergic nature. Since ENL often occurs in the lepromatous patient during treatment, destruction of mycobacteria with a probable release of *M. leprae* antigens may be indicated in the pathogenesis of the syndrome. Analogies have been made between ENL and the Jarisch-Herxheimer reaction frequently encountered in the treatment of syphilis (37). Histologically, the lesions of ENL resemble those of the Arthus phenomenon with deposition of antigen-antibody complexes in vessel walls with resulting complement fixation eventuating in a leukocytoclastic vasculitis. An important characteristic of ENL is that there is no change in leprosy classification during the course of the reaction (68,70,212), and there is no clinical evidence of extension of the disease or new lepromatous lesions (37).

Recommended treatment for ENL are steroids, clofazimine, or thalidomide when not contraindicated (15,68).

**Pathogenesis of ENL:**

Erythema nodosum leprosum occurs only on a substrate of lepromatous leprosy (37,68,70). Patients with this form of leprosy demonstrate the immunological dichotomy of a split anergy having hypergammaglobulinemia with varying degrees of depressed CMI (170). The pathogenic mechanism for the initiation of ENL has not been described. Proposed
mechanisms for the initiation of ENL may be divided into three broad categories: immune complexes (either tissue complexes or circulating immune complexes) with activation of classical complement pathways; activation of the alternate pathway of complement without participation of immunoglobulins; or some perturbation in control mechanisms at the cellular level (68,148).

Lepromatous leprosy patients carry a high bacillary load which provides chronic antigen stimulation. ENL is often precipitated during treatment where the bacterial index (BI) and morphological index (MI) drop as the patient responds favorably to therapy. Chronic antigen stimulation in lepromatous patients provides a stimulus for continued antibody production. A cyclical production of antibody, particularly IgM, may result from such persistent antigen stimulation (68,70,212). Polyclonal immunoglobulinemia has been observed in leprosy patients both with and without ENL. New patients also tend to present with elevated immunoglobulin levels. IgM-type antibodies may decline with treatment, while IgG- and IgA-type antibodies may remain elevated (30,68,70,170,216).

Because the histological picture of ENL is so similar to that of the Arthus phenomenon, it has long been postulated that ENL is mediated by the deposition of antigen/antibody complexes and subsequent activation of the classical complement pathway. ENL also has systemic features very like that of serum sickness, suggesting the
presence of circulating immune complexes (17). An early indication of immune complex formation was provided by the demonstration of cryoglobulins in lepromatous patients (113). Cryoglobulins were present in 97% of those without ENL, 100% of those with untreated ENL, and 58% of those with ENL on steroid therapy. These cryoglobulins were shown to be of a mixed type and anti-IgG activity was demonstrated in the IgM component (18). It was postulated that autologous IgG may become autoantigenic following reaction with a primary antigen. This altered IgG may then stimulate the host immune system to respond by producing a corresponding IgM antibody. These complexes (IgG plus anti-IgG -IgM) could then be instrumental in the induction of ENL (18).

Deposits of immunoglobulin and complement have been demonstrated by immunofluorescence in the lesions of approximately 55% of ENL patients (212,214). The deposits were perivascular and located in areas corresponding to the infiltration of polymorphonuclear neutrophils (PMNs). Soluble mycobacterial antigen was detected in some of these complexes, which were sometimes located within the walls of blood vessels. It was suggested that the lesions of ENL are caused by these complexes, either formed locally (Arthus) or present in the circulation.

Deposits of immunoglobulin alone have been demonstrated in the skin of lepromatous patients (29,73,147). Specific, fine granular deposits of
IgM have been detected at the dermal-epidermal junction (29) and also in the dermal collagen and elastic fibers of lepromatous patients (147). Two possibilities were suggested for the etiology of these deposits: tissue-bound IgM may be specific antibody to mycobacterial antigens, or anti-mycobacterial antibody may cross react with basement membrane antigen. More recently, skin biopsies from reactive nodular lesions were shown to contain IgG (100%) as well as IgM (58%) in deposits at the dermal-epidermal junction (73). It is still not known if these deposits are specific antibodies to mycobacterial antigens or autoantibodies.

Circulating immune complexes have been detected in the sera of leprosy patients by Clq precipitation in gel (122,160). Presence of these complexes was shown to correlate with the clinical condition; complexes were present in 33-70% of the lepromatous patients without ENL, 76% of the lepromatous patients with active ENL, 20% of the tuberculoid patients, and only 3% of normal controls. It was suggested that localized complexes might mediate the ENL lesions (Arthus), while circulating immune complexes might be associated with the systemic symptoms of ENL. Clq precipitation and gel assays are not without controversy. Tung and associates have demonstrated that Clq also reacts with DNA and bacterial antigens - the results of such tests for the detection of immune complexes should be interpreted with caution in patients with infectious diseases (201).
Clq binding assays do not give false-positive reactions with organisms or DNA (104). Using these methods, it has been shown that circulating immune complexes exist in the sera of 82% of leprosy patients with uncomplicated lepromatous leprosy, 80% of lepromatous patients with ENL, and 58% of tuberculoid patients (16). Differences also exist between leprosy patients in endemic and nonendemic areas (205). In endemic areas, patients across the leprosy spectrum can demonstrate circulating immune complexes. Circulating immune complexes are not demonstrable in TT and BT patients from nonendemic areas. Thus, circulating immune complexes are probably neither specific for ENL nor related specifically to leprosy. Reliable interpretations of immune complexes in leprosy patients will probably only be available when examinations are made using antigens specific for M. leprae.

Using C3d levels as a measure of complement catabolism evidence for increased complement activation in patients with ENL has been reported (16,205). Two sources of C3d were postulated: immune complexes or C3d fragments diffusing from extravascular sites of immune complex deposition and complement activation (16). During ENL, C3d levels were high but returned to normal once the clinical signs of ENL had subsided (205). Thus, it was suggested that ENL lesions are mediated by complement and that elevated C3d levels are highly specific for ENL.
Londono (109) found C3d deposits in vessel walls, but was unable to demonstrate immunoglobulin and suggested that the alternate pathway of complement played an important role in ENL. Other investigators have noted a fall in the C3 level and an elevation in the Ba (breakdown product of factor B) in ENL (168). These workers proposed that during the initial phase of acute ENL there is a sudden release of *M. leprae* antigens into circulation which first initiates the classical complement pathway and, subsequently, the alternate complement pathway. Overall, there is little agreement about the role of complement in the initiation of ENL.

Mshana, et al. (126) found mycobacterial antigen in all biopsies from lepromatous patients (with or without ENL), while only 23% of ENL patients were positive for immunoglobulin or complement deposits. Both ENL and lepromatous patients without ENL had foamy macrophages with large amounts of mycobacterial antigen. In ENL, these macrophages were said to rupture and release antigens into the extracellular spaces. Absence of immunoglobulin and C3 in the presence of extracellular antigen in ENL was taken to indicate that mycobacterial antigen is more important than antigen-antibody complexes for the initiation of ENL (158,159).

The presence of large quantities of *M. leprae* antigens provides chronic stimulation to the partially responsive immune system of lepromatous patients. Turk has suggested
that the continued stimulation of these cells might induce a population of B suppressor cells capable of modulating T cells, as well as those that develop into conventional antibody producing cells (200). Bach (9) and Wallach (209) used monoclonal antibodies to show that lepromatous patients with recent ENL have a decreased number of T suppressor cells in the peripheral blood and an increased number of T helper cells. In mixed lymphocyte cultures these cells were noted to have an elevated response to mitogen. The helper-suppressor ratio was normal in both tuberculoid patients and nonreactional lepromatous patients.

It was postulated that the imbalance between T cell subsets contributed to the occurrence of ENL in lepromatous patients. Later, Mshana (125) showed an increase in T suppressors and the peripheral blood of lepromatous leprosy patients and a transient acute inversion of the helper/suppressor ratio during ENL. The transient decrease in T suppressors could be due either to decreased production of T suppressors or to increased sequestration of those cells in tissues (161). The mechanism by which these cells may affect regulation in ENL is as yet unknown. However, other cell types seem to be affected during ENL. Cytotoxic T cells have been shown to be defective in lepromatous patients without ENL and to become functional in patients during ENL (189). This deficiency could be either part of a reactional process or part of the underlying T cell abnormality of lepromatous leprosy.
Humphres, et al. (76) has described depressed natural killer (NK) cell activity in ENL patients, and the patients' own monocytes were seen to be responsible for the depression of NK activity.

The various theories as to the induction of ENL are not mutually exclusive, and a number of mechanisms may interact. Regardless of the exact sequence of events and whether ENL is cellular or immune-complex initiated, the common denominator is the presence of PMNs in ENL lesions. It is obvious that this is the cell that directly mediates the tissue damage. What is not known is the mechanism by which PMNs are recruited.

Thalidomide has been shown to be effective in the treatment of ENL. Understanding the mechanism of action of thalidomide in halting the immunological and/or inflammatory events in ENL might lead to a better understanding of the immunopathology of ENL reactions. Thalidomide has been shown to prevent PMN chemotaxis (13, 69), to stabilize lysozomal membranes, and to antagonize certain chemical mediators of inflammation (histamine, serotonin, acetylcholine, and prostaglandin E-2 and F-2 alpha) (71). Therapeutic doses of thalidomide decrease the generation of toxic oxygen intermediates by PMNs (120). Shannon (176) demonstrated a selective decrease in IgM in leprosy patients during treatment of ENL with thalidomide, as well as an inhibition of de novo IgM synthesis in mice. It was proposed that IgM antibody is
the relevant immunoglobulin-initiating immune complex formation in ENL. Recent studies using the T-independent antigen DNP-Ficoll suggest that thalidomide either has no effect or slightly augments the IgM antibody response to this antigen. Therefore, thalidomide appears to selectively suppress the IgM response to T-dependent antigens (175). IgM is primarily intravascular (80%) and the maximum complement fixing ability of IgM and soluble antigen/antibody complexes is formed when the antibody is in excess. Therefore, favorable conditions for a type III hypersensitivity reaction with IgM and complement fixation occur intravascularly. Complex formation at the vascular epithelium can first occur as antigen diffuses from tissue spaces (32). As initial vascular damage occurs permeability increases, allowing penetration of IgM into the vessel wall since more complexes may form as more antigen is encountered. Perhaps only when ENL is examined in the light of antibodies, antigen and immune complexes specific for antigens of M. leprae will the pathogenesis of the reaction be understood.

Serology:

Resistance to leprosy is primarily dependent on the cell-mediated immune system, but M. leprae does stimulate an apparently nonprotective humoral response (1,203). Serology holds hope to aid the early diagnosis and management of leprosy cases. Antibody levels in leprosy
patients have been reported to reflect the different clinical forms of the disease (91) and to change with alteration of the antigen load in chemotherapy (91,152,197). A good review of serological attempts in leprosy has been prepared by Melsom (130). Generally, complement fixation, passive hemagglutination, double diffusion in gels, indirect immunofluorescence (IFA), gel precipitation, radioimmunoassay (RIA), and enzyme-linked immunosorbent assays (ELISA) have been used to detect antibodies in leprosy patients.

Complement fixation tests were introduced for the diagnosis of several infectious diseases around 1900. Eitner (46) showed that antigen preparations from human lepromas could fix complement with sera from leprosy patients. Sera from lepromatous leprosy patients showed high reactivity in the complement fixation tests, while sera from tuberculoid patients showed poor or no reactivity (46). Based upon these and similar findings using different test systems for antibodies in sera from leprosy patients, paucibacillary tuberculoid leprosy patients have been classified as "poor antibody responders" and patients with multibacillary lepromatous leprosy, "good antibody responders" (26). This antibody response is also reflected in increased immunoglobulin concentration (40) and in increased frequency of autoantibodies, like rheumatoid factors, anti-thyroid antibodies, and anti-nuclear antibodies (149). Additionally, components from several
mycobacterial and diphtheroid species could fix complement with sera from leprosy patients (40). Therefore, the test lacked specificity and only had use in diagnosis of lepromatous leprosy (41,45).

Mycobacterial antigen-antibody reactions were first examined by hemagglutination tests in 1948 (119). Sheep red blood cells (SRBC) were found to be the best carrier cells (139), and better results were obtained when SRBC were sensitized with M. tuberculosis than with M. leprae (7). Low positive titers often resulted in sera from normal controls. Higher titers were noted in tuberculoid leprosy patients, and the highest titers were manifested by lepromatous leprosy patients (106). More recently, there have been attempts to coat tanned SRBC with M. leprae from armadillos. Specificity was enhanced by absorption of test sera with M. tuberculosis and M. vaccae. Absorbed lepromatous sera still contained antibodies which agglutinated the red blood cells (83). However, these sera also reacted with M. lepraemurium, M. bovis, M. phlebesin, M. gastri, M. gordonae and M. nonchromogenicum by immunodiffusion methods (162). In some lepromatous leprosy patients a decrease in titer was found in sera taken some months after sulfone treatment was begun (162). An increase in titer could also be demonstrated when sera were taken during an ENL reaction (42). The hemagglutination test primarily confirmed the previous findings from the
complement fixation test but also lacked specificity in its determinations.

The Rubino reaction was described in 1926 as a sedimentation reaction between formalin-treated SRBC and sera from leprosy patients. Sera from patients with diseases other than leprosy could not sediment such treated SRBC (43,166). The Rubino test was mostly positive with sera from lepromatous patients and negative with sera from tuberculoid patients. Until recently, the test has been used in Latin America. However, the Rubino reaction can be inhibited by antigens from several mycobacterial species and is positive during renal failure (5,164). The test is not specific for M. leprae, and the value seems limited.

Gel precipitation methods were first applied using lepromin and serum from lepromatous patients in 1957 (138). This technique, applied in double diffusion schema and crossed immunoelectrophoresis, has been used extensively in the characterization of antigens of M. leprae. These methods indicate that leprosy patients may respond differently to a number of cross reactive antigens of M. leprae. Diffusion techniques are not suitable for quantitation of antibody reactions, and the heterogeneity of patient response make them unsuitable for the diagnosis of leprosy and will not be discussed.

The indirect fluorescent antibody (IFA) technique was first used to demonstrate antibodies to M. leprae and M. tuberculosis in 1961 (128). Leprosy patients had positive
reactions with both *M. lepraе* and *M. tuberculosis*. The technique was further developed by Abe (2). To aid specificity, Abe applied a series of absorbtions with cardiolipin, lecithin, BCG, and *M. vaccae*. After absorbtion, diluted serum was applied to smears of *M. lepraе* obtained from leprosy patients or armadillos (3). To enhance reaction by removal of lipids and exposing protein antigens, smears were treated with carbon-tetrachloride and trypsin. Reading was accomplished with a special combination of fluorescent filters and based on the amount of total fluorescence emitted by a single organism. Antibodies were detected in 100% of the lepromatous leprosy patients and 80% of the tuberculoid patients. Antibodies were not detected in normal control groups but were found in household contacts. The IFA test was also modified for use and presumptive identification of *M. lepraе* organisms (114). Recently, it has been shown that sera absorbed by the scheme of Abe could also react with *M. lepraemurium*, *M. bovis*, *M. nonchromogenicum*, *M. phlebesin*, *M. gordonae* and *M. gastri* (49). Therefore, exposure to environmental mycobacteria and BCG may have influence on the results of these studies. IFA is not specific for *M. lepraе*, and interpretations are subjective. The test has not received wide application in other laboratories.

Radioimmunoassay (RIA) has been used both competitively and in solid-phase assays with antigen
systems not specific for M. leprae. The first RIA in leprosy used the antigen 60 from BCG. Antibodies were detected in 25 of 29 lepromatous patients and in 11 of 20 tuberculoid patients. However, BCG vaccination is in wide use and antibodies to this antigen would not be reliable indicators of leprosy infection (63). Using gel diffusion to examine the sera of leprosy patients, Harboe noted a specific line of precipitation more commonly formed by reaction with fractions of sonicated whole M. leprae. Using this antigen 7 mixture in RIA, antibodies were detected in 80% of the lepromatous patients and occasionally detected in tuberculoid patients (66).

Levels of antibodies in lepromatous patients were noted to fall after the first year of dapsone therapy (135). In examining the sera from armadillos, antibodies to antigen 7 became detectable in experimental infections with M. leprae only after establishment of the disseminated infection (49). Owing to the mixture of antigens in the preparation, it was again necessary to absorb test sera with other mycobacterial species to enhance specificity (64). At this time it is uncertain if the absorption scheme adequately removes all cross reacting antibodies with M. leprae. The requirements for absorption may vary in different populations depending on the mycobacterial flora (130). While the original RIA was designed using Staphlococcal Protein-A as a precipitant of antibodies reacting with iodinated fractions of whole M. leprae, the
The phenolic-glycolipid-1 antigen is chemically defined and readily available from the tissues of experimentally infected armadillos (21,77). Although its phthiocerol core is shared with *M. kansasii*, cross reactions with the specific trisaccharide epitope have not been described (33). The nonaqueous nature of this antigen originally complicated immunologic investigations. To demonstrate antigenicity in gel diffusion assays required incorporation into liposomes (22,141). Although complicating other aspects, this hydrophobicity especially
recommends the antigen as an immunosorbent in solid-phase assays. Organic solvents and detergents were not useful in dispersing phenolic-glycolipid-1 to solid phases. They either reacted with the sorbent material or would not release the antigen for hydrophobic binding.\(^{(78)}\) Dispersing the antigen, either by deacylation of its lipid side chains or sonication of the native lipid, proved most successful and today, a number of investigators have developed ELISA assays \(^{(44,107)}\). At this time, most reports in the literature concern the specificity of the antigen. The specificity of the antigen lends well to sero-epidemiologic studies, and there is hope that the antigen may be used in the early serodiagnosis of leprosy \(^{(20,24)}\). Phenolic-glycolipid-1 also offers a unique opportunity to examine *M. leprae*-specific humoral responses in leprosy patients. The following studies use this antigen as a mechanism to examine *M. leprae*-specific humoral responses in leprosy patients and in armadillos for both sero-epidemiology and management.
DETECTION OF IgM CLASS ANTIBODIES
IN ARMADILLOS
TO THE PHENOLIC-GLYCOLIPID-1 ANTIGEN OF M. LEPRAE.

1. DEVELOPMENT OF A SEROLOGIC TEST

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Chapter 3: Abstract

An enzyme-linked immunosorbent assay (ELISA) using antisera specific for human IgM and the phenolic-glycolipid-1 (Phen-Gl-1) antigen of \textit{M. leprae} has been developed for immune status assessment of armadillos. Human and armadillo IgM express sufficient serologic cross reactivity to allow the use of commercially prepared anti-human IgM to detect armadillo IgM antibodies. Testing plasma from 76 armadillos having a known experimental inoculation history with \textit{M. leprae} showed the assay to have high sensitivity and specificity. IgM anti-Phen-Gl-1 antibodies were detected both before and after the appearance of acid-fast bacilli in peripheral blood, buffy coat cells of experimentally infected armadillos, and up to 36 months after the experimental infection. Armadillos not experimentally infected with \textit{M. leprae} (4/20) also had detectable IgM antibodies to Phen-Gl-1. These antibodies were absorbable with whole \textit{M. leprae}, suggesting that wild armadillos may have an antibody prevalence rate as high as 20%. The assay has application for sero-epidemiology and the monitoring of experimental infections with \textit{M. leprae} in armadillos.
The nine-banded armadillo (Dasypus novemcinctus) is the most common host for the in vivo propagation of Mycobacterium leprae (97) and is an animal model for the study of leprosy (194). Captive breeding of armadillos is generally unsuccessful, and animals must be taken from the wild for use in scientific studies. Wild armadillos have been reported to harbor a mycobacterial infection that is indistinguishable from M. leprae (210). To avoid contamination of cultured strains and assure similarity of immunological responses in model systems, recently captured animals must be screened to assess their naiveté to M. leprae.

Serology is a traditional means of screening animals for infectious experiences, but the serology of M. leprae has been most difficult. M. leprae bears a number of antigens which cross react with other mycobacterial species (103,136). The insectivorous, burrowing armadillo habitually encounters a wide variety of common soil mycobacteria and likely develops antibodies to many cross reacting antigens of M. leprae. Previous serological assays for M. leprae antibodies in armadillos have utilized traditional Staphylococcal-A radioimmunoassay methodologies with iodinated fractions of the sonicated whole leprosy bacillus (64,66). Consequently, they lacked both specificity and the ability to distinguish antibody isotypes (179). Recently, a chemically defined phenolic glycolipid antigen (Phen-Gl-1) of M. leprae has been
isolated from the tissues of experimentally infected armadillos. The antigen has not been found in other mycobacterial species and is apparently specific for *M. leprae* (22,33,78). Others have reported the development and utilization of enzyme-linked immunosorbent assays (ELISA) for antibodies to Phen-Gl-1 in human sera (24,78). We report here the development of an ELISA that detects IgM-class antibodies in armadillo serum reacting with the Phen-Gl-1 antigen of *M. leprae*.

**MATERIALS AND METHODS**

**Anti-armadillo serum**: Rabbit anti-armadillo serum globulins was a gift of Dr. T. E. Wilson, University of Southeastern Louisiana (Lafayette, Louisiana).

**CIEP**: Crossed immunoelectrophoresis (CIEP) was performed by the method of Clark and Freeman (34). Serum was electrophoresed in 1% agarose gel with 0.025 M tricine buffer, pH 8.6, with constant voltage of 20 volts/cm (BioRad 3000/300, Richmond, CA). Electrophoresis proceeded for 50 min in the first dimension when a second slab was cast that contained either second antibody conjugate or anti-armadillo immunoglobulins. Electrophoresis was performed in the second dimension for 60 min before the slabs were washed in normal saline and stained with Coomassie blue to visualize antibody reactions.

**Antigen**: Native purified phenolic glycolipid-1 (Phen-Gl-1) was prepared by Dr. Patrick Brennan (Colorado
State University) and obtained through contract with the National Institute of Allergy and Infectious Diseases (Dr. Darrel Gwinn, Project Officer).

**Experimentally infected control group:** Armadillos at the National Hansen's Disease Center are captured from the wild and housed for the production of *M. leprae*. They are periodically bled, both pre- and post-experimental inoculation with *M. leprae*, to examine for the presence of acid-fast bacilli in peripheral blood, buffy coat cells. Plasma from these bleedings is separated and stored at -80°C for future use. Seventy-six such samples were used for this study. Twenty of the samples were from animals before inoculation with *M. leprae*; one of the 20 was from an armadillo originally presenting from the wild with an acid-fast bacterial infection that was indistinguishable from that caused by *M. leprae* in that the bacilli oxidized D-DOPA (144,145) and their acid fastness was extracted with pyridine (115). The remaining 56 samples were from armadillos that had been inoculated with *M. leprae* from 4 to 43 months before bleeding. Thirty-one of the 76 armadillos had acid-fast bacteremia at the time of bleeding. Two of these 76 sera were from animals that had resisted challenge with *M. leprae*, as shown by having no signs of infection 28 months after inoculation with at least $1 \times 10^8$ viable leprosy bacilli.
ELISA: ELISA-IgM vs. Phen-Gl-1: An enzyme-linked immunosorbent assay (ELISA) was performed according to the method of Voller and Bidwell (79). Specifically, native phenolic-glycolipid-1 (Phen-Gl-1) was mixed with 0.05 M carbonate buffer, pH 9.5, and suspended via sonication with a 3 mm probe at 70 watts for 1 minute (Ultrasonics, Inc., Plainville, NY). The suspended antigen was diluted in the same buffer to a concentration of 2 μg in 50 μl the volume which was added to each well of a 96 well polyvinyl flat bottom microtiter plate (Cooke Labs, Alexandria, VA). The plates were sealed with Parafilm (American Can Co., Greenwich, CT) and incubated over night at 37°C in a water bath. After incubation, the wells were washed 3 times for 5 minutes each wash with 200 μl of wash buffer consisting of 0.067 M phosphate-buffered saline (PBS), pH 7.2, containing 1% bovine serum albumin (BSA) (Fraction V; Sigma, St. Louis, MO). After washing, the wells were blocked by the addition of 100 μl of 0.067 M PBS, pH 7.2, containing 5% BSA and incubated at 37°C for 1 hr. The wells were then washed as before and 50 μl of each serum sample diluted in wash buffer was added to duplicate wells in an effective dilution of 1:180. The plates were sealed, and incubated at 37°C for 1.5 hr when they were removed, and washed as before. Horseradish peroxidase conjugated IgG fraction of goat anti-human IgM (μ specific) (Cappel, Cochranville, PA) optimally diluted (1:4000) in 50 μl of
wash buffer was added to each well. The plates were then sealed and incubated for 2 hr at 37°C, when they were removed and washed as before. After washing, 50 μl of a solution containing 0.4 mg/ml ortho-phenylene-diamine with 0.002% H₂O₂ in 0.1 M sodium acetate buffer, pH 5.5, was added to each well. The plates were incubated at room temperature for 20 min when the reaction was stopped with the addition of 50 μl 9 N HCl. The plates were read for absorbency at 488 nm on a Beckman DU8B spectrophotometer. Control absorbent values from wells containing serum but not receiving antigen were subtracted from serum plus antigen wells. The mean absorbent value for each sample was calculated for use in statistical analysis.

**Optimal dilutions:** The optimal dilution of serum was defined to be that lowest dilution which did not yield nonspecific absorption greater than 2 times the substrate control when reacted in blank wells. Serum was diluted 1:100, 1:400 by two-fold dilutions in the ELISA wash buffer and reacted with an optimal dilution (1:4000) of conjugate (see below).

The optimal concentration of the *M. leprae* Phen-Gl-1 antigen was determined by checkerboard titration. Positive and negative armadillo sera were selected on the basis of known inoculation history with *M. leprae*. Conjugate and sera were used at constant dilutions in an ELISA wash buffer at 1:4000 and 1:180, respectively. The Phen-Gl-1 antigen was coated in microtiter plates at concentrations
of 100 μg/ml with two-fold dilutions to 3 μg/ml. Coating and incubations were followed as described before. The optimal concentration was defined as that highest dilution in antigen excess resulting in the greatest difference in absorbency readings between positive and negative sera.

To determine the optimum range of conjugate for use in the assay, the conjugate, horseradish peroxidase IgG fraction of goat anti-human IgM (mu specific), was diluted two-fold from 1:100 to 1:32000 in an ELISA wash buffer. A checkerboard titration was performed across the wells of a polyvinyl microtiter plate coated with serial dilutions of human IgM (Cappel, Cochranville, PA) at 100, 50, 10, 5, 1, and 0.1 ng/well. Following incubation and wash procedures as described above, the optimum range was defined as those dilutions of conjugate yielding best linearity in absorbency readings between 1 and 10 ng of human IgM. Optimal dilution of conjugate was later determined in checkerboard titration across the wells of a polyvinyl microtiter plate coated with 50 μg/ml of Phen-Gl-1 and reacted with positive and negative serum as delineated before. The optimal dilution of conjugate was defined to be the highest dilution which also yielded the greatest difference in absorbency readings between the positive and negative sera.

**Mycobacterial species:** Cultivable mycobacterial species (M. scrofulaceum, M. phlei) were inoculated to Lowenstein-Jensen (L-J) slants and grown to confluency.
The organisms were harvested using Hanks' balanced salt solution (HBSS) and pelleted via centrifugation 2000 x g for 10 minutes. They were subsequently washed 5 times with HBSS and enumerated (182). The organisms were aliquoted to separate tubes such that each tube contained $1 \times 10^9$ bacteria. They were pelleted by centrifugation and the supernatants discarded. *M. leprae* were propagated in armadillos and harvested from tissues by manual teasing into 1 gm pieces. Tissue and bacteria were separated by differential centrifugation in HBSS and the supernatants were saved. Supernatants containing *M. leprae* were then pelleted by centrifugation and washed 5 times with HBSS. Organisms were enumerated and aliquoted to separate tubes such that they would contain $1 \times 10^9$ *M. leprae* after supernatants were removed.

**Absorption of sera:** Selected serum samples were absorbed with the mycobacterial species. Sera diluted 1:180 in 400 μl of ELISA wash buffer were added to the tubes containing $1 \times 10^9$ pelleted mycobacteria. Pellets were resuspended by gentle shaking every 15 min while the tubes were incubated for 1 hr at 37°C. The tubes were then held overnight at 4°C, and the suspensions were clarified by centrifugation before use. The absorbed supernatant serum was then assayed by the ELISA as described previously.
RESULTS

Cross reactivity of human and armadillo IgM:

Human IgM and armadillo IgM are serologically cross reactive. Crossed immunoelectrophoresis (CIEP) of armadillo whole serum into a second dimension containing rabbit anti-armadillo globulins produced a typical CIEP pattern with multiple lines of precipitation (Fig. 3.1a). While CIEP into a second dimension containing peroxidase conjugated goat anti-human IgM (mu chain specific) demonstrated a single precipitation line of slow migration like that of IgM (Fig. 3.1b), other armadillo serum proteins were not detected with the anti-human IgM peroxidase conjugate.

Optimization of ELISA: For the ELISA, the lowest serum dilution was selected which did not result in excessive nonspecific binding of immunoglobulins to the carrier surface. As shown in Figure 3.2a, serum dilutions less than 1:180 resulted in nonspecific binding to the solid phase $\geq 2.5$ times the substrate control. Sera diluted 1:180 avoided this nonspecific binding and was selected as optimal.

Optimal proportions of antigen and antibody aid in the sensitivity of the assay. As shown in Figure 3.2b, coating wells with 2 $\mu$g of Phen-Gl-1 resulted in antigen excess while allowing maximum dilution of the antigen in the system.
A maximum conjugate dilution of 1:4000 yielded the greatest difference of absorbency readings between positive and negative samples (Figure 3.2c). This dilution did not result in nonspecific absorption of conjugate to the solid phase. This conjugate dilution also showed linearity of reaction between 1 and 10 ng of human IgM (data not shown).

**ELISA of control group:** Seventy-six plasma samples from armadillos housed at the National Hansen's Disease Center armadillo farm were assessed. The results are illustrated in a frequency histogram (Figure 3.3a) and a scattergram (Figure 3.3b). When the sample was examined, over 10 equal class intervals of 200 absorbent units each, an apparent bi-modal distribution was revealed (Figure 3.3a). When the population was more closely scrutinized in a scattergram, the apparent separation in absorbency readings along the continuum creating two groups was shown to be distinct. Multivariate discriminant analysis showed this separation to be statistically significant ($p < 0.001$; $N=52, N=23$) (36,192) (Fig. 3.3b). The boundaries of the two groups as demarked by two standard deviations around their respective means created an equivocal zone. It was considered that points falling within the equivocal zone could not reliably be ascribed to either of the two groups.

The data points were then pooled and analyzed according to their known experimental inoculation history with *M. leprae* (i.e., months post-inoculation) and appearance of acid-fast bacilli (AFB) in peripheral blood,
buffy coat cells. Points falling above the equivocal zone were predominantly (48/52) from armadillos known to have been experimentally inoculated with *M. leprae* (Figure 3.4). All samples from armadillos with acid-fast bacteria in their buffy coat smears appeared above the equivocal zone. Of those points falling below the equivocal zone, 16 of 23 animals were not experimentally inoculated with *M. leprae*, while the remaining 7 were known to have been inoculated with *M. leprae*. One animal was considered to be resistant to the infection. The single point falling within the equivocal zone also was derived from an animal considered resistant. Thus, the points falling above the equivocal zone represent sera from animals with detectable IgM antibodies to Phen-Gl-1, and were considered "positive". Those below the zone did not have detectable IgM antibodies to Phen-Gl-1 and were considered "negative". Animals not manifesting AFB appear on both sides of the zone, while those demonstrating AFB grouped only above the zone. Regressional analysis was performed for AFB positive and AFB-negative animals with time after experimental inoculation. Both groups regressed significantly and their slopes appeared to be different, showing the ELISA to be semi-quantitative and suggesting a difference in the rates of antibody development in the two groups.

Four armadillos not experimentally inoculated with *M. leprae* also appeared in the positive group. One of these armadillos presented from the wild with an acid-fast
bacterial infection indistinguishable from \textit{M. leprae}. Absorption of these four sera with whole \textit{M. leprae} significantly reduced ($P = 0.001$, paired t test) the antibody reactivity. Absorption with \textit{M. scrofulaceum} and \textit{M. phlei} did not significantly alter the antibody reactivity of these sera (Figure 3.5).

**ELISA IgM vs. Phen-Gl-1 quality parameters:** The serological parameters of sensitivity, specificity, efficiency, and predictive value for positives (79) were calculated for the ELISA IgM vs. Phen-Gl-1 based on the available data. As shown in Table 3.1, the assay correctly detects IgM antibodies in an armadillo with a known previous experience with \textit{M. leprae} 100\% of the time (predictive value of positive). With a 95\% confidence, the assay offers a sensitivity of 89\%, a specificity of 100\%, with an overall efficiency of correctly identifying the infectious experience of an armadillo 90\% of the time.

**DISCUSSION**

These data indicate that an enzyme-linked immunosorbent assay incorporating cross reactive antisera for human IgM and the Phen-Gl-1 antigen correlates with, and therefore may be used to assess the immunological experience of armadillos with \textit{M. leprae}. The ELISA-IgM vs. Phen-Gl-1 avoids problems of nonspecific reactivity found in assays using undefined antigen mixtures, and allows detection of
IgM class antibodies to *M. leprae*. The assay is an efficient (90%) tool for serological investigations of *M. leprae* in armadillos, and affords sensitivity (89%) and apparent specificity (100%) heretofore unreported for serological assays in leprosy (196).

Serological cross reactivity of human IgM has been noted in several other species including cows, monkeys and sharks (74). Use of commercially prepared reagents in immunoassays allows for their wider application in other laboratories. Armadillo antisera are not commercially available, but armadillo IgM has substantial serological cross reactivity with human IgM. Fortunately, therefore, commercially prepared anti-human IgM second antibody enzyme conjugates are satisfactory for use in ELISA assays for armadillo IgM antibodies.

Serological screening tests must distinguish samples as positive or negative. To do so, the descriptive limits of positive and negative must be defined and the assays must be optimized to avoid nonspecific reactivity. By screening sera from 76 armadillos of known inoculation history with *M. leprae*, statistical limits for positive and negative have been derived for the ELISA IgM vs. Phen-Gl-1. Sera from this group may now be used as standards in subsequent screenings to allow the interpretation of new results as positive or negative.

Screening of the test group showed that 87% (49/56) of the animals experimentally infected with *M. leprae* had
detectable IgM antibody to the Phen-Gl-1 antigen. Of the 7 armadillos experimentally inoculated with M. leprae but not demonstrating detectable antibody, two were from resistant animals. Approximately 10% of the armadillos experimentally infected at the National Hansen's Disease Center are resistant to M. leprae - they do not succumb to lepromatous disease (95). Resistance in leprosy is governed by cell-mediated immunity (96). Humoral immunity may not be mustered in these resistant animals. Further investigation is needed to determine if serological monitoring of armadillos may be useful in the early identification of resistant and susceptible animals.

Antibody profiling of armadillos experimentally infected with M. leprae will aid in their development as animal models in leprosy research (60,99). Previous efforts to detect M. leprae antibodies in armadillos have exercised traditional RIA methodologies with iodinated fractions of the sonicated whole bacillus (66). Those reports indicated that IgG antibodies to M. leprae became detectable only simultaneously with the appearance of AFB in the peripheral blood (64). Experimentally infected armadillos both with and without acid-fast bacteria in the peripheral blood had detectable IgM antibodies to the Phen-Gl-1 antigen. These ELISA data suggest that IgM antibodies to the Phen-Gl-1 antigen are detectable well in advance of overt systemic infection. These antibodies appear to be chronically synthesized over the course of
infection and are present in comparatively high concentrations until the animals are sacrificed. Therefore, IgM class antibodies to the Phen-Gl-1 antigen appear to be good indicators of subclinical or early leprosy, and may be the more appropriate isotype for sero-epidemiological investigations.

Other investigators using the Phen-Gl-1 antigen in ELISA assays of human sera have reported approximately a 4 to 5% apparently "false-positive" rate in presumed negative control groups. These reports are based on resulting ELISA absorbancies above 0.1 in traditional ELISA methodologies used on the sera of individuals from leprosy nonendemic areas and exhibiting no clinical signs of leprosy (23). Wild armadillos have been reported to harbor a mycobacterial infection indistinguishable from M. leprae (210). Our test group contained 4/20 samples from 4 recently captured armadillos not inoculated with M. leprae which yielded ELISA absorbancies statistically interpretable as positive. When these sera were absorbed with M. leprae, their ELISA reactivity was significantly reduced, while absorption with M. phlei and M. scrofulaceum did not significantly alter their ELISA reactivity. Presumably, therefore, these are true positive reactions suggesting that modern day wild armadillos in Louisiana may have an M. leprae antibody prevalence rate as high as 20%.
In conclusion, the serology of mycobacterial diseases is complicated by the plethora of cross reacting antigens found among the various species. Isolation of an apparently species-specific antigen for *M. leprae* has allowed the development of serologic tests with high specificity. Antibody profiling of experimentally infected armadillos will aid their development as animal models for leprosy and better descriptions of *M. leprae* infections in wild armadillos, as measured serologically, may well provide a better understanding of the transmission of leprosy in humans.

ACKNOWLEDGMENTS

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Table 3.1
Elisa-IgM v. Phen-Gl-1 quality parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ratio</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>53 (true+) + 6 (false-)</td>
<td>89</td>
</tr>
<tr>
<td>Specificity</td>
<td>16 (true-) + 0 (false+)</td>
<td>100</td>
</tr>
<tr>
<td>Efficiency</td>
<td>53 (true+) + 16 (true-)</td>
<td>90</td>
</tr>
<tr>
<td>Efficiency of Positive</td>
<td>53 (true+) + 0 (false+)</td>
<td>100</td>
</tr>
</tbody>
</table>

True+ = Absorbance > 710 and known inoculation with M. leprae or absorbable Phen-Gl-1 antibody.

True- = Absorbance < 560 and no known history with M. leprae.

False+ = Absorbance > 710 with no known history with M. leprae and unabsorbable antibody to Phen-Gl-1

False- = Absorbance < 710 with known inoculation of M. leprae.

* Known resistant animals are not included.
Table 3.2
Legends to figures

Figure 3.1 Crossed immunoelectrophoresis of whole armadillo serum. A) Second dimension contains rabbit anti-armadillo immunoglobulins. B) Second dimension contains horseradish peroxidase conjugated IgG fraction goat anti-human IgM (mu chain specific).

Figure 3.2 Optimal dilutions for ELISA. 3.2A) Absorbance (488 nm) resulting from nonspecific absorption of IgM to polyvinyl microtiter plates at various dilutions of armadillo serum. 3.2B) Absorbance (488 nm) resulting from positive pooled control serum reacting with various concentrations of Phen-G1-I coated to wells of polyvinyl microtiter plates showing optimal dilution to be 25 ug/ml. 3.2C) The difference in absorbance (488 nm) between control positive and negative antisera reacted with 25 ug Phen-G1-I coated microtiter plates detected by various dilutions of horseradish peroxidase conjugated IgG fraction goat anti-human IgM (mu chain specific). SUBSTRATE ABS = substrate absorbance.
Figure 3.3 Distribution of ELISA absorbance of 76 armadillos with known experimental inoculation history. 4A) Frequency histogram showing number of samples occurring in each of 10 absorbance classes and demonstrating apparent bimodality. 4B) Scatter plot of ELISA absorbances showing significant difference, $p < 0.001$) in means of the two groups and demarcation of equivocal zone.

Figure 3.4 Regression of ELISA absorbances according to the presence (*) or absence (o) of acid-fast bacilli (AFB) in peripheral blood. * = acid-fast positive; o = acid-fast negative; R = resistant armadillos.

Figure 3.5 ELISA absorbance (488 nm) of 4 suspected false positive armadillo sera after absorption with M. leprae, M. scrofulaceum and M. phlei. Equivocal zone is demarcated by solid horizontal bars.
Figure 3.1
Crossed immunoelectrophoresis of Armadillo Whole Serum in the First Dimension against (a) Anti-Armadillo Whole Serum; or (b) Horseradish Peroxidase Conjugated Anti-Human IgM (mu specific) in the Second Dimension.
Figure 3.2 ELISA OPTIMAL DILUTIONS
Histogram (3.3b) and Scattergram (3.3b)

Figure 3.3 ELISH-198 vs Phen-C1 Frequency

Histogram (3.3b) and Scattergram (3.3b)

Figure 3.3 ELISH-198 vs Phen-C1 Frequency

Histogram (3.3b) and Scattergram (3.3b)

Figure 3.3 ELISH-198 vs Phen-C1 Frequency

Histogram (3.3b) and Scattergram (3.3b)

Figure 3.3 ELISH-198 vs Phen-C1 Frequency
Figure 3.4 Regression according to presence(−) or absence(−−) of AFB in peripheral blood.
Figure 3.5  ELISA-IgM vs. Phen-G1-1 Specificity Absorbtions
CHAPTER 4

DETECTION OF IgM CLASS ANTIBODIES IN ARMADILLOS TO THE PHENOLIC-GLYCOLIPID-1 ANTIGEN OF M. LEPRAE.

2. EVALUATION OF THE ORIGIN OF M. LEPRAE INFECTION IN WILD ARMADILLOS

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Chapter 4: Abstract

Using an enzyme-linked immunosorbent assay that detects armadillo IgM class antibodies to the apparently species-specific phenolic-glycolipid-1 (Phen-Gl-1) antigen of *M. leprae*, a retrospective serological survey was performed. Sera from 182 armadillos taken in the years 1960 to 1964 and predating the use of armadillos in leprosy research were tested. Antibodies to the Phen-Gl-1 antigen were detected in 17 of the sera. Absorption with whole *M. leprae* significantly reduced the antibody activity of the sera. Absorption with *M. avium*, *M. intracellulare*, *M. scrofulaceum*, BCG, *M. dierhoferi*, *M. phlei*, *M. rhodesiae*, *M. terrae*, *M. kansasii*, and two new mycobacterial isolates from armadillos 8152 and 8480 failed to significantly alter the antibody activity of these sera. These antibodies presumably indicate that wild armadillos experienced infections with *M. leprae* as early as 1961.
Man has traditionally been thought to be the only natural host of *Mycobacterium leprae*, the etiological agent of leprosy. However, studies beginning in 1968 demonstrated that the nine-banded armadillo (*Dasypus novemcinctus*) is susceptible to experimental infection with *M. leprae* (97). Since 1975, a number of reports have appeared which establish that wild armadillos harbor a mycobacterial infection indistinguishable from *M. leprae* (14,184,185,210,211). Recently, five cases of human leprosy have been reported in individuals from Texas, whose only contact with *M. leprae* may have been through long-term close association with armadillos (110). Thus, the discovery of *M. leprae* in the wild armadillo has not only raised many questions about the transmission and host range of leprosy, but identified a possible public health risk.

How the armadillo became infected with *M. leprae* is central to a better understanding of the transmission of leprosy. Generally, three possible answers have been proposed: a) *M. leprae* may be natural to the armadillo and indigenous to the population (212) or armadillos may have acquired leprosy either by b) association with infected humans or c) inadvertent environmental contamination from experimentally infected armadillos sometime since 1968 (183).

We recently developed an enzyme-linked immunosorbent assay (ELISA) that detects armadillo IgM antibodies to the apparently species-specific phenolic glycolipid antigen
We report here an evaluation of the post-1968 environmental contamination hypothesis for the origin of *M. leprae* infections in wild armadillos.

**MATERIALS AND METHODS**

**Wild armadillo sera 1960-1964:** A total of 182 armadillo serum samples were collected by Adams and Roth in 1960-1964 as part of a survey for leptospires in wild animals (165,166,167). The samples were tested for leptospiral antibodies and stored at -10°C for approximately 12 years when they were deposited in the Louisiana State University School of Veterinary Medicine serum bank and stored at -79°C. Information relative to sex, time, date, location of capture, known antibody titers and previous users was recorded for each serum.

**Leptospiral serology:** In 1983 the Louisiana Veterinary Medical Diagnostic Laboratory (LVMDL) repeated leptospiral antibody titration on armadillo sera that had been found to be positive in 1960-1964. The modern leptospiral method employed by the LVMDL uses a microtitration technique with commercially prepared antigens and control sera. The initial dilution for a positive sample by the LVMDL procedure was 1:100. The procedure originally used by Adams and Roth in 1960-1964 was a tube agglutination method with individually prepared
antigens and 1:25 as a minimum dilution for a positive serum.

**Antigen:** Native, pure phenolic-glycolipid-1 (Phen-Gl-1) was prepared by Dr. Patrick Brennan (Colorado State University) and obtained through contract with the National Institute of Allergy and Infectious Disease, Dr. Darrel Gwinn, Project Officer.

**ELISA-IgM vs. Phen-Gl-I:** An enzyme-linked immunosorbent assay (ELISA) was performed according to the method described previously (198).

**Mycobacterial species:** Mycobacterial strains 8152 and 8480 are "difficult to grow" isolates arising from the tissues of armadillos. They were a gift from Dr. Francoise Portaels (Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium). Cultivable mycobacterial species (M. avium, BCG, M. diernhoferi, M. intracellulare, M. kansasii, M. phlei, M. rhodesiae, M. scrofulaceum, M. terrae, and Portaels' bacilli 8152 and 8480 were inoculated to Lowenstein-Jensen (L-J) slants and grown to confluency. The organisms were harvested using Hanks' balanced salt solution (HBSS) and pelleted via centrifugation at 700 x g. They were subsequently washed 5 times with HBSS and enumerated (182). Bacterial suspensions were diluted to contain 1 x 10⁹ organisms per ml in 0.067 M phosphate buffered saline (PBS) with 1% bovine serum albumin (PBS/BSA), pH 7.2. **M. leprae** were propagated in
armadillos, harvested as described previously (215) and likewise diluted in PBS/BSA.

**Absorption of sera:** Enumerated bacterial suspensions were aliquoted to disposable microfuge tubes (Beckman Instruments, Palo Alto, CA) in 200 µl volumes at a concentration of $1 \times 10^9$ organisms/ml. Serum samples with ELISA absorbances interpretable as positive were diluted 1:180 in PBS/BSA and added in 200 µl volumes to tubes containing mycobacteria or PBS/BSA alone. Sera and bacteria were mixed by gentle shaking every 15 min as the tubes were incubated for 1 hr at 37°C. The tubes were then held overnight at 4°C, and the suspensions were clarified by centrifugation on a Beckman Microfuge model B for 5 min before use. The absorbed serum was assayed in the ELISA as described above.

**RESULTS**

**Wild armadillo serum 1960-1964**

**History:** Armadillo serum samples collected in 1960-1964 originated from a six-parish area of the Atchafalaya Basin in south central Louisiana. The area is located west of the Mississippi River and north approximately 40 miles from both the Gulf South Research Institute in New Iberia, Louisiana, and the National Hansen's Disease Center in Carville, Louisiana. The Basin is a wild swamp area with mixed habitat and hosts abundant wildlife. It is similar in habitat but different in location to the site of the original report of *M. leprae* in
wild armadillos by Walsh, et al. (210). The Atchafalaya holds soils common to the Louisiana delta region with pH ranging from mildly acidic (5.0) to slightly alkaline (7.7) (188). The specific capture site of each armadillo could be identified within 100 yards and the collection area is mapped in Figure 4.1. Histological sections of kidneys from 50 of these armadillos were examined in 1973 by Stewart and Crowell (195). Acid-fast bacilli were not observed in the tissues examined. Liver tissues were not examined.

Utility of pre-susceptible sera after 20 years of storage: Armadillo sera taken in 1960-1964 were evaluated for usability after 20 years of storage. Leptospiral serology was repeated on the 11 known positive samples. The modern leptospiral technique employs a microtitration method with commercially prepared antigens and standardized antisera. The technique of 20 years ago utilized tube agglutination with individually prepared antigens. Initial dilutions of the two techniques also differ and, therefore, direct correlation of titer results between the two methodologies is not possible. However, positive and negative correlations are still feasible. As shown in Table 4.1, 7 of the 11 armadillo sera known to be positive for Leptospira species retained their antibody activity after 20 years of storage. None of the tested sera changed in antibody specificity to the individual leptospiral antigens. A proportion (4/11) of the sera lost
all detectable antibody activity over the 20 years of storage.

**ELISA:** The 182 wild serum samples taken 4-8 years before initial susceptibility studies of armadillos to *M. leprae* were performed in 1968, were screened in the ELISA IgM vs. Phen-Gl-1. The absorbances of these samples were inspected in a frequency histogram which displayed a positive skew (Figure 4.2). Resulting absorbances were judged according to the definitions of positive and negative as previously determined (198), and 17 samples exhibiting absorbance readings above 0.720 were classed as presumptive positive.

**Absorption of antibody activity:** The 17 presumed positive sera were absorbed with whole *M. leprae* and 11 other heterologous mycobacterial species. The ELISA was repeated and the absorbance results were analyzed by paired t-test for significance. As shown in Figure 4.3, as a group the 17 sera were significantly (p < 0.001) depleted of detectable antibody reactive with Phen-Gl-1 after absorption with whole *M. leprae*. As a group the ELISA absorbances were not significantly altered when absorption was performed with other mycobacterial species. ELISA absorbances of a highly positive control (+) serum derived from an armadillo experimentally infected with *M. leprae* followed an identical pattern. Absorbances of a negative control (-) serum remained unchanged.
DISCUSSION

The discovery of *M. leprae* in the wild armadillo created controversy concerning the origin, transmission and host range of leprosy. Whether *M. leprae* is indigenous to armadillos, or wild armadillos acquired *M. leprae* either by association with infected humans or contact with *M. leprae* contaminated materials some time since the 1968 armadillo susceptibility trials, has been widely debated (183). We evaluated the 1968 environmental contamination hypothesis by means of a retrospective serological survey. The sera from 182 wild armadillos taken in years (1960-64) pre-dating the 1968 susceptibility trials were examined for IgM antibodies to the Phen-Gl-1 antigen of *M. leprae*.

Antibody to the apparently species-specific phenolic glycolipid antigen (Phen-Gl-1) of *M. leprae* (22,33,78) was detected in 17 of 182 sera taken from wild armadillos in 1960-64. Reactivity of these antibodies was significantly reduced by absorption with whole *M. leprae*. This reactivity was not significantly altered by absorption with 11 other mycobacterial species tested (Fig. 4.3). The absorption scheme sought to eliminate antibodies that might have been induced by some "Original Mycobacterial Sin" (4). Members of the MAIS antigenic complex (12) (*M. avium, M. intracellulare, M. scrofulaceum*) and BCG may potentiate the immunologic response to *M. leprae* (11) and may hold common antigens (137). Additionally, common soil mycobacteria,
represented here by *M. diernhoferi*, *M. phlei*, *M. rhodesiae*, and *M. terrae*, may also induce this phenomenon. *M. kansasii* is known to share a common phthicerol core antigen with *M. leprae* (81) and other, as yet unrecognized, mycobacterial species (represented here by Portaels' bacilli 8152 and 8480 isolated from armadillo tissue) could also function in this area. Failure by these species to absorb the antibody activity to the apparently species-specific Phen-Gl-1 antigen suggests that those antibodies were elicited as a result of exposure to *M. leprae*.

Long-term storage of serum samples may result in protein deliquescence, antibody aggregation, or loss of antibody reactivity (185). To assure the utility of these 182 sera after 20 years of storage, leptospiral antibody activity was reassessed. Since technical methodologies of leptospiral serology have changed in 20 years, direct correlation of titer results was not possible. Results could only be judged as positive or negative. A proportion (4/11) of these sera apparently had lost leptospiral antibody activity over the years of storage (Table 4.1). Since the true proportion of sera that had lost reactivity could not be discerned, it was not possible to describe prevalence rates in the 1960-1964 population. However, none of the serum samples previously negative for *Leptospira* species antibodies was found to be positive after 20 years of storage. These sera maintained their
specificity and had utility for seroepidemiological investigation.

Adams and Roth collected these sera along with visceral tissues from 182 armadillos in the Atchafalaya Basin of south central Louisiana during the years 1960-1964. Samples were collected as part of a survey for leptospires in Louisiana wildlife. After testing, the materials were stored frozen for future reference. In 1973, Stewart and Crowell (195) examined sections of kidneys from 50 of these armadillos but failed to detect acid-fast bacteria. Armadillos experimentally infected with *M. leprae* at the National Hansen's Disease Center rarely express acid-fast bacteria in renal tissues (173), and the failure of Crowell and Stewart to detect acid-fast bacteria in these kidney tissues should not be considered unusual.

Certain environmental associations have been made with leprosy infections in humans, and there appear to be trends to higher prevalence rates in humid climates (80). Kazda recently reported isolation of a leprosy-like organism from a peat bog in Norway having a pH of 5.5 (92). Such mildly acidic soil pH conditions have been identified as optimal for isolation of some mycobacterial species from soils (10,190). The Atchafalaya Basin is a wild swamp area and soils of the region are typical to the delta of Louisiana, ranging in pH from 5.0 to 7.7 depending upon drainage. No reports have been made of attempts to isolate *M. leprae* or
leprosy-like organisms from soils of the Atchafalaya, but such investigations may be warranted.

Antibodies may be regarded as highly specific indicators of infectious experiences. The presence of antibodies to the apparently species-specific Phen-Gl-1 antigen in armadillos during the years 1960-1964 suggests that M. leprae was present in the wild population before the armadillo was used as an experimental host for M. leprae cultivation. Presumably then, the original infection of wild armadillos with M. leprae could not have resulted from any inadvertent environmental contamination associable with the 1968 armadillo susceptibility trials or any subsequent experimentation with armadillos. The true origin of M. leprae infection in the wild armadillo remains an important scientific question. If the infection was not the result of accidental contamination, how have armadillos contracted leprosy? An understanding of the origin of this infection in armadillos may have considerable impact on our understanding of the transmission of leprosy in humans.

ACKNOWLEDGMENTS

We would like to thank Troy McMannus, Louisiana Veterinary Medicine Diagnostic Laboratory, for technical assistance in leptoserology, and Mrs. Renee Painter for her excellent secretarial assistance. This work has been supported in part by a grant from the American Leprosy Missions.
Table 4.1
Reassessment of 1960-1964 wild armadillo leptospiral serology after 20 years of storage

<table>
<thead>
<tr>
<th>Sample</th>
<th>Canicola pre</th>
<th>Grippota&lt;sup&gt;a&lt;/sup&gt; pre</th>
<th>Pomona pre</th>
<th>Icteroh&lt;sup&gt;b&lt;/sup&gt; pre</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>.</td>
<td>400</td>
<td>400</td>
<td>.</td>
</tr>
<tr>
<td>14</td>
<td>100</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>100</td>
</tr>
<tr>
<td>34</td>
<td>.</td>
<td>100</td>
<td>.</td>
<td>100</td>
</tr>
<tr>
<td>36</td>
<td>.</td>
<td>100</td>
<td>400</td>
<td>.</td>
</tr>
<tr>
<td>96</td>
<td>400</td>
<td>.</td>
<td>.</td>
<td>100</td>
</tr>
<tr>
<td>106&lt;sup&gt;d&lt;/sup&gt;</td>
<td>.</td>
<td>.</td>
<td>25 200</td>
<td>100</td>
</tr>
<tr>
<td>118</td>
<td>.</td>
<td>100</td>
<td>.</td>
<td>100 200</td>
</tr>
<tr>
<td>165&lt;sup&gt;d&lt;/sup&gt;</td>
<td>.</td>
<td>.</td>
<td>25 100</td>
<td>.</td>
</tr>
<tr>
<td>208</td>
<td>.</td>
<td>100</td>
<td>100</td>
<td>.</td>
</tr>
<tr>
<td>209&lt;sup&gt;d&lt;/sup&gt;</td>
<td>.</td>
<td>100 100</td>
<td>25 100</td>
<td>25 100</td>
</tr>
</tbody>
</table>

<sup>a</sup>=Grippotyphosa  
<sup>b</sup>=Icterohemorrhagea

Four culture positive when captured

<sup>c</sup> L. pomona  
<sup>d</sup> L. autumnalis
Table 4.2

Legends to figures

Figure 4.1 Map of general capture area where 182 wild armadillos were taken by Adams and Roth in the years 1960 to 1964.

Figure 4.2 ELISA absorbances of 182 wild armadillos taken in the years 1960 to 1964 plotted in a frequency histogram of equal class intervals. Figure demonstrates positive skew of sample group.

Figure 4.3 ELISA absorbances of presumed positive wild armadillo sera collected in the years 1960 to 1964 and absorbed with various mycobacterial species. Absorption with whole M. leprae resulted in significant (paired-t; p < 0.001) reduction in ELISA absorbances. Absorption with other mycobacterial species not significant. Point(+) = control positive serum; point(-) = control negative serum; point 1-17 = serum samples from 1960-1964 wild armadillos.
Figure 4.1
Map of 1960 to 1964 Armadillo Capture Area.
Figure 4.2 Frequency Histogram ELISA-IgM v. Phen-G1-1
Figure 4.3 1960-64 Positive Sera Absorbed with Mycobacterial Species
Figure 4.3 1960-64 Positive Sera Absorbed with Mycobacterial Species
Figure 4.3  1960-64 Positive Sera Absorbed with Mycobacterial Species
CHAPTER 5

THE NINE BANDED ARMADILLO

AS AN ANIMAL MODEL TO STUDY THE
TRANSMISSION OF LEPROSY

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Chapter 5, Abstract:

Sera from 216 wild armadillos of two parishes in south central Louisiana and Florida were tested by ELISA for IgM antibodies to the phenolic-glycolipid-1 antigen of M. leprae. Antibodies were not detected in sera from Florida armadillos (0/30). Louisiana armadillos had an antibody prevalence rate averaging 12.5% (23/186). Antibody prevalence rates between Louisiana and Florida were significantly different. Antibody prevalence rates of armadillos of the two Louisiana parishes were similar. The clinical disease rate was estimated to be 2.7% for Louisiana armadillos by histologic examination to detect disseminated acid-fast bacteria. The population characteristics and environmental conditions of the Louisiana leprosy positive habitat were described. Future comparisons of prevalence rates in armadillos of different habitats will aid in understanding the transmission of leprosy and further develop the armadillo as an animal model for leprosy research.
The transmission of leprosy is not yet understood. It is suspected to be obligately transmitted from man to man and may require long term close contact with infected humans (58). However, other mechanisms have been implicated. Environmental conditions have been described that correlate with increased prevalence rates of leprosy (80) and Kazda has reported isolation of an *M. leprae*-like organism from a sphagnum bog in Norway (92). Mycobacteria sharing certain phenotypes with *M. leprae* may be found in nature. The role of environment or environmental microorganisms as pathogens or additional factors influencing the transmission of leprosy has not been determined (190,191). A better understanding of these influences may aid in the control of leprosy.

Epidemiologic studies on the transmission of leprosy in man are complicated by low susceptibility and mobility of the human population. However, leprosy is zoonotic in the nine-banded armadillo (*Dasypus novemcinctus*) (14,185,186,210,211), and approximately 90% of armadillos are susceptible to leprosy (95,96). Thus armadillos may be used as an animal model to study the transmission of leprosy.

To effectively use the armadillo as an animal model, large groups must be sampled with sensitive screening techniques and relevance must be given to the environment and population characteristics of the groups studied. Previous investigations of leprosy in wild armadillos have...
relied on histologic examinations to detect disseminated acid-fast bacteria. The leprosy-like disease has been confirmed in various locales and prevalence rates have been estimated to range from 0 to 25% (14,185,186,210,211). Such disparate estimates may be the result of insensitivity of the histologic screen, population characteristics or environmental conditions.

We recently developed an enzyme-linked immunosorbent assay (ELISA) that detects armadillo IgM antibodies to the phenolic-glycolipid-1 (Phen-Gl-1) antigen of *M. leprae* (198). This antigen is chemically defined and apparently species specific for *M. leprae* (22,33,78). Antibodies to this antigen may be detected in the absence of disseminated disease (198). We report here preliminary data assessing IgM anti-Phen-Gl-1 antibody prevalence rates in wild armadillos of Louisiana and Florida, and a description of a leprosy positive armadillo habitat.

MATERIALS AND METHODS

**Serum and histologic samples:** Armadillos were captured in 2 parishes of south central Louisiana: Iberville and Point Coupee. A total of 131 armadillos from Iberville parish were taken by local trappers in the vicinity of Sunshine, Louisiana and brought to the National Hansen's Disease Center (NHDC) in Carville, Louisiana. At the NHDC armadillos were sampled by venipuncture and snips
of ears were taken for histologic examination to detect acid-fast bacilli (AFB). A total of 55 different armadillos in Point Coupee parish were sampled in the field near Krotz Springs, Louisiana and along a tract where 2 armadillos sero-positive for leprosy had been taken in the years 1960-64 (see preceding chapter). Field serologic samples were taken in capillary tubes after the excision of a toenail. A sample of ear was taken before the animals were released.

Serum samples from 30 Florida armadillos were collected by Dr. E. Storrs (Florida Institute of Technology) and provided as a gift through the National Institute of Allergy and Infectious Diseases (Dr. Darrel Gwinn, Program Officer).

**Population Density:** Armadillos in Point Coupee Parish were captured over a 10 mile stretch of levee bordering the Atchafalaya River and comprising an area of approximately 200 ha (500 acres). Using a system of capture-mark-recapture the total population was estimated by the Lincoln-Peterson method: 

$$\frac{[(\text{total number of marked and unmarked animals taken on the final trapping day}) \times (\text{total number of animals marked})]}{(\text{total number of previously marked animals recaptured on the final trapping day})}$$

The population density was the ratio of total population/area sampled.

**Antigen:** Native purified phenolic-glycolipid-1 (Phen-Gl-1) was prepared by Dr. Patrick Brennan (Colorado
State University) and obtained through contract with the National Institute of Allergy and Infectious Diseases (Dr. Darrel Gwinn, Project Officer).

**ELISA:** Enzyme-linked immunosorbent assay (ELISA) was performed according to the method described previously (198).

**Histology:** Tissue was prepared by standard methods as reported previously and examined for the presence of acid-fast bacteria (AFB) in nerves (86).

**RESULTS**

**Capture area:** Armadillos were taken from 2 parishes in Louisiana: Iberville and Point Coupee. The parishes border, but specific capture areas were some 40 miles apart. The Point Coupee site was a tract along the Atchafalaya River while Iberville bordered the Mississippi River. Both parishes provided excellent habitat and hold abundant wildlife. A total of 49 animals were marked in the Point Coupee area and 1 was recaptured on the final trapping day. Using the Lincoln-Peterson method of capture-recapture (108) the population density in Point Coupee was estimated to be 5 per 7 acres. The density in Iberville was not calculated. Average climatic conditions of the 2 parishes were also similar (Table 5.1): annual rainfall 54.9", relative humidity 73.7%, yearly temperature 69°F. Neither of the parishes had been inundated by floods.
since 1973 (35), but areas where armadillos were taken were poorly drained. Soils of the two areas were only slightly different with Convent types (coarse-silty, mixed, thermic aeric fluvaquents, pH 5.6 to 8.4) predominating Point Coupee; and Commerce types (fine-silty, mixed, thermic aeric fluvaquents, pH 5.6 to 7.8) predominating Iberville (186,187). The conditions occupied by Florida armadillos were unknown.

**Antibody prevalence:** A total of 186 serum samples taken from armadillos in Louisiana (131 Iberville, 55 Point Coupee) and 30 samples from Florida armadillos were screened by ELISA for IgM antibodies to Phen-Gl-1. The resulting absorbances were judged according to definitions of positive and negative as previously determined for the ELISA (198) (Fig. 5.1). A total of 23 (12.5%) Louisiana armadillos had detectable IgM antibodies to Phen-Gl-1. Antibodies were detected in 10.7% (14/131) of the armadillos from Iberville parish and 16.3% (9/55) of the armadillos from Point Coupee. None of the Florida armadillo sera had detectable IgM antibodies to Phen-Gl-1. Antibody prevalence rates from the various groups were compared for significant differences by Chi square (Table 5.2). Armadillos from Iberville and Point Coupee were not significantly different. However, antibody prevalence rates of Louisiana and Florida armadillos were significantly different (p < 0.05).
Histologic prevalence: Leprosy prevalence rates for Louisiana armadillos were also described by histologic methods. Acid-fast bacteria (AFB) were detected in 2.7% (5/186) of the Louisiana animals. Armadillos from both parishes presented with detectable AFB in ear tissues: 3 Iberville, 2 Point Coupee. Each armadillo with detectable AFB also had positive serologic findings. However, 18 armadillos from both parishes had detectable IgM antibodies to Phen-Gl-1 without manifesting detectable AFB.

DISCUSSION

To effectively use the armadillo as an animal model for the transmission of leprosy; large populations must be sampled with sensitive screening methods and relevance must be given to population characteristics and environment of the groups under study. Previous studies of leprosy in the wild armadillo have relied on histologic methods to identify infected animals. Histology alone may lack adequate sensitivity to describe leprosy prevalence rates in armadillos. Acid-fast bacteria may only be detected in armadillos once infection has established and disseminated. Therefore histology is a measure of clinical disease. Examination of ear tissues from Louisiana armadillos indicated clinical leprosy in only 2.7% (5/186) of the animals examined. Previous reports using histology to describe the prevalence of leprosy in Louisiana armadillos
have also averaged only 4.9% (116). However, the ELISA suggest armadillos may have a greater experience with leprosy. Prevalence rates for IgM antibodies to Phen-Gl-1 averaged 12.5% in Louisiana armadillos. This lipid antigen promotes a predominate long term IgM response (24,198,217). Antibodies may be elicited after exposure to antigen or early in the course of infection. Thus, the ELISA is a better reflection of sub-clinical and antigenic experiences. Used in combination, these methods should provide adequate sensitivity to investigate leprosy in the wild armadillo.

The population characteristics operated by different groups of armadillos may influence their observable prevalence rates. If leprosy is obligately transmitted from armadillo to armadillo, then groups with greater density might have higher prevalence rates, those with lower density, lower rates. Armadillos along a research site in the Atchafalaya Basin of Point Coupee Parish were estimated to have a density of 5 per 7 acres with an antibody prevalence rate of 16.3%. In 1952, the only previous estimate of density for armadillos in another part of Louisiana was 1 per 7 acres (48). Future comparisons of antibody prevalence rates in other groups will also require new calculations of population density to better understand the transmission of leprosy.

If environmental factors influence transmission then armadillos in different environments may have different
prevalence rates of leprosy. The two groups of Louisiana armadillos inhabited similar environments. Antibody prevalence rates of the two groups also were similar. Environmental conditions of high relative humidity and warm vegetation temperatures were implicated to influence increased historical human leprosy prevalence rates in Norway (80). Louisiana armadillos had significantly higher antibody prevalence rates than Florida armadillos. Antibodies to Phen-Gl-1 were not detected in Florida armadillos. Naturally acquired leprosy has not been reported in the Florida armadillo. Louisiana and Florida armadillos appear to be equally susceptible to experimental infection with *M. leprae* (93); but, the populations may occupy different environments and until recently may not have been in contact. Armadillos in Louisiana slowly migrated here from Texas and Mexico. Armadillos may have been accidentally introduced to Florida in 1925 following the escape of several pairs from a private zoo in Cocoa. Zoologists report that over the years these 2 populations have eventually merged (48,75,111), and except for leprosy, there are no discernible differences between the groups. The actual reason for these observed differences in antibody prevalence rates between Louisiana and Florida armadillos is uncertain but may be due to differences in population characteristics, environment or availability of contact with an infectious source.
In conclusion, leprosy is zoonotic in wild armadillos and these animals may be used to study the transmission of leprosy. Serologic screening combined with histology should provide suitable sensitivity for these investigations. While for the most part armadillos are susceptible to leprosy, significant differences in prevalence rates have been observed. These differences may be due to environmental factors, population characteristics or availability of contact with an infectious source. Future comparisons of leprosy prevalence rates from armadillos in different environments and/or operating different population characteristics will help decipher the transmission of leprosy in armadillos and aid our understanding of the transmission of leprosy in man.

Acknowledgements

We express our appreciation to Larry Aiken, Ian Ross and Frank Austin for their help in the field studies conducted for this project.
<table>
<thead>
<tr>
<th>Month</th>
<th>Relative humidity am &amp; pm</th>
<th>Rainfall inches</th>
<th>Rain days</th>
<th>Temp °F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jan.</td>
<td>85 &amp; 65</td>
<td>4.58</td>
<td>9</td>
<td>51.7</td>
</tr>
<tr>
<td>Feb.</td>
<td>54 &amp; 59</td>
<td>4.97</td>
<td>9</td>
<td>54.2</td>
</tr>
<tr>
<td>Mar.</td>
<td>85 &amp; 57</td>
<td>4.59</td>
<td>9</td>
<td>60.8</td>
</tr>
<tr>
<td>April</td>
<td>88 &amp; 56</td>
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<td>7</td>
<td>68.2</td>
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<td>May</td>
<td>90 &amp; 57</td>
<td>4.82</td>
<td>8</td>
<td>74.8</td>
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<td>June</td>
<td>91 &amp; 58</td>
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<td>9</td>
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<tr>
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</tr>
<tr>
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<td>3.95</td>
<td>7</td>
<td>60.0</td>
</tr>
<tr>
<td>Dec.</td>
<td>86 &amp; 63</td>
<td>4.99</td>
<td>10</td>
<td>54.0</td>
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<td>Average month</td>
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<td>8.9</td>
<td>67.9</td>
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</tr>
<tr>
<td>Total</td>
<td>50.72 &amp; 107</td>
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Table 5.2
Chi Square Comparison of Phen-G1-1 Antibody Prevalence Rates of Louisiana and Florida Armadillos

<table>
<thead>
<tr>
<th>Number</th>
<th>Iberville</th>
<th>Point Coupee</th>
<th>Louisiana</th>
<th>Florida</th>
</tr>
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<tbody>
<tr>
<td>Negative</td>
<td>113</td>
<td>46</td>
<td>163</td>
<td>30</td>
</tr>
<tr>
<td>Positive</td>
<td>13</td>
<td>9</td>
<td>23</td>
<td>0</td>
</tr>
</tbody>
</table>

Percent Positive
10.3 16.3 12.5 0

Iberville v. Point Coupee $X^2=1.31$ (not significant)
Louisiana v. Florida $X^2=4.15$ ($p<0.05$)
Table 5.3
Legend to figure

Figure 5.1. Scattergram of ELISA absorbances for IgM antibodies to Phen-Gl-1. Absorbance coded X 1000. Horizontal bars mark equivocal zone previously determined for the ELISA. Absorbances above 710 on graph are positive for IgM antibodies, below 580 negative. Point Coupee (Louisiana) N=55, Iberville (Louisiana) N=131, Florida N=30, (Louisiana N=186).
Figure 5.1  ELISA-IgM v. Phen-G1-1 Wild Armadillos
CHAPTER 6

EVALUATION OF ELISA-IgM v. PHEN-GL-1 FOR USE IN MONITORING EXPERIMENTAL INFECTIONS OF ARMADILLOS WITH MYCOBACTERIUM LEPRAE

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95
Chapter 6, Abstract:

An enzyme-linked immunosorbent assay (ELISA) that detects IgM antibodies to the apparently species specific phenolic-glycolipid-1 antigen of *M. leprae* was evaluated for its efficacy as a management tool in the in vivo propagation of *M. leprae* in armadillos. Screening armadillos both pre and post experimental infection indicated that IgM antibodies became detectable on average after 186 days and remained so over the course of infection. ELISA absorbances from sera taken at sacrifice showed good correlation with the liver yield of *M. leprae*. An ELISA absorbance of greater or less than 1.2 at sacrifice could be used to predict satisfaction with the harvest 97% of the time. ELISA profiles of resistant armadillos showed that IgM antibody production was irregular or absent, and the ELISA could be used to predict resistance in armadillos both early and late in experimental infections. The ELISA successfully identified armadillos with naturally acquired leprosy and indicated that these armadillos may also have unusual antibody profiles that would make them unsuitable for controlled immunologic studies. IgM antibodies were detectable earlier and more reliably than previously used histologic monitoring measures. The ELISA is less labor intensive and non-invasive, making it a useful tool in the management of experimental leprosy infections in armadillos.
**Mycobacterium leprae** has not been cultured on artificial media and investigators must rely on *in vivo* propagation as a source of bacilli. The nine-banded armadillo, *Dasypus novemcinctus*, is well developed as a host for propagation of *M. leprae*, and studies have begun that would use this animal as a model for the pathogenesis of leprosy (86,93,94,97,194). Effective use of animals in research on infectious diseases requires monitoring of the host over the course of experimental infection. Previous methods to monitor experimental infections of *M. leprae* in armadillos have commonly relied on histologic techniques (97). These techniques are labor intensive and invasive to the animal. Serologic assays are less laborious and offer hope for greater efficiency in management of experimental infections. Previous serologic attempts to monitor *M. leprae* antibodies in armadillos have relied on traditional *Staphylococcal-A* radioimmunoassay methodologies with iodinated fractions of the sonicated whole leprosy bacillus. Using undefined antigen mixtures these assays lacked specificity and only detected *M. leprae* antibodies simultaneous with the appearance of a disseminated infection (151,160). We recently developed an enzyme linked immunosorbent assay (ELISA) that detects armadillo IgM antibodies to the chemically defined and apparently species-specific phenolic-glycolipid-1 (Phen-Gl-1) antigen of *M. leprae* (198). We report here an evaluation of this
ELISA for applications in the management and investigation of experimental *M. leprae* infections in armadillos.

**MATERIALS AND METHODS**

**Seroologic and Histologic Samples:** Armadillos at the National Hansen's Disease Center are captured from the wild and housed for the production of *M. leprae*. They are bled approximately every 90 days, both pre and post experimental inoculation to examine for the presence of acid-fast bacilli (AFB) in peripheral blood. Histologic samples of ear tissues are also taken and examined for AFB to monitor dissemination of disease. Plasma from these bleedings is separated and stored frozen for future reference. A total of 457 such plasma samples from 210 different armadillos were used in this study. Armadillos were at varying stages of infection with an average intravenous inoculum of $5 \times 10^8$ *M. leprae* derived from human leproma and armadillo passage. Infection status ranged from not yet inoculated to sacrificed. Status and clinical history, according to the date of each serum sample, were recorded as part of the routine production of *M. leprae*.

**Antigen:** Native purified phenolic glycolipid-1 (Phen-Gl-1) was prepared by Dr. Patrick Brennan (Colorado State University) and obtained through contract with the
National Institute of Allergy and Infectious Diseases (Dr. Darrel Gwinn, Project Officer).

**ELISA:** Enzyme-linked immunosorbent assay (ELISA) was performed according to the method previously described (198).

**Histology:** Histologic evaluations were performed using standard methods described previously (215).

**RESULTS**

**Comparison of ELISA with yield of M. leprae:**

The ELISA absorbancies of sera from 35 armadillos sacrificed after experimental infection with an average intravenous inoculum of 5 x 10^8 M. leprae were compared to their liver yield of AFB. Armadillo harvests yielding less than 1 x 10^7 organisms per gram of liver tissue are considered unsatisfactory for production purposes (203). In this comparison, all armadillos with unsatisfactory harvests were defined to yield 1 x 10^7 organisms per gram of liver tissue. Actual yields from these armadillos may have been less. Armadillos yielding greater than 1 x 10^7 organisms per gram of liver tissue were compared using the log of their actual yield. As shown in Figure 6.1, there is a significant correlation between ELISA absorbance and the yield of M. leprae from liver tissues at sacrifice (r = 0.7261) (p < 0.001). The majority (25/26) of armadillos yielding satisfactory harvests (greater than 1 x
$10^7$ *M. leprae* per gram of liver tissue) had ELISA absorbances greater than 1.2 at sacrifice. None of the armadillos with unsatisfactory harvests ($1 \times 10^7$ or less *M. leprae* per gram of liver tissue) had ELISA absorbances greater than 1.2 at sacrifice. Using the criteria of ELISA absorbances greater or less than 1.2 the satisfaction with *M. leprae* yield at sacrifice was predictable 97% (34/35) of the time.

**ELISA profile of resistant armadillos:**

Serial samples from four armadillos subsequently shown to be resistant to experimental infection with *M. leprae* were screened by the ELISA-IgM v. Phen-Gl-1 (198). As shown in Figure 6.2, the resulting ELISA absorbances were compared with their days post-infection. Two armadillos failed to produce detectable IgM antibody to the Phen-Gl-1 antigen in any of the samples tested. IgM antibody to the Phen-Gl-1 antigen was detectable in a third armadillo at 246 days post-infection; but that antibody waned after 452 days. A fourth armadillo also showed an irregular pattern of IgM antibody response to the Phen-Gl-1 antigen. Acid-fast bacteria were not detected by histologic examination of ear tissues from these 4 armadillos.

**ELISA profile of armadillos with wild-type infection:**

Armadillos not experimentally infected with *M. leprae* but obtained from the wild with ELISA absorbances greater than 0.71 are interpretable as positive in the ELISA-IgM v.
Phen-Gl-1 (198). Presumably these animals have acquired leprosy naturally in the wild (210). Retrospective samples were available for 8 such armadillos in this study (Fig. 6.3). Their ELISA absorbances before experimental infection ranged from 0.743 to 1.155. In one animal (1) a disseminated acid-fast bacterial infection was found prior to experimental infection with M. leprae by histologic examination of ear tissues. This armadillo was removed from the colony. The ELISA absorbances of this animal increased until the time of removal. A second (3) animal also developed detectable AFB in ear tissues within 26 days of experimental infection with M. leprae. ELISA absorbances showed a slight but steady increase until this animal was also removed from the colony. Three other armadillos (2,4,5) had a steady and progressive increase in ELISA absorbances after experimental inoculation with M. leprae. One (2) developed signs of a disseminated infection as early as 97 days post inoculation. The two other armadillos (4,5) did not express detectable AFB in ear tissues over the time these samples were taken. Three other armadillos (6,7,8) presenting from the wild with ELISA absorbances interpretable as positive showed a different pattern of ELISA absorbances after inoculation with M. leprae. Absorbances declined such that IgM anti-Phen-Gl-1 antibodies were not detectable. Disseminated AFB were detected in ear tissues of one animal (8) at 97 days and it was removed. ELISA absorbances of
the other 2 animals (6,7) eventually increased again to detectable levels, and in one (7) AFB were detected in the blood at 404 days.

**Kinetics of antibody development:**

A total of 404 serum samples from 198 armadillos at various stages of infection were tested by ELISA to determine the time course of development of IgM antibodies to the Phen-Gl-1 antigen. Armadillos presenting from the wild with postive ELISA were not included. Sera from sacrificed armadillos with unsatisfactory yields were also not included. The actual wild experience, as well as the eventual possibility of resistance for all armadillos, could not be determined; and some wild infected and resistant armadillos are no doubt included in this group of 198. The mean ELISA absorbances were calculated as a mean of each group of sera taken in 82 day intervals post infection. Means were plotted according to the last day in that interval (Fig. 6.4). Using the statistical definitions previously derived for interpreting positive and negative in this ELISA (198), the average day an armadillo might be expected to elicit detectable IgM antibodies to the phenolic-glycolipid-1 was approximately 200 days post experimental infection. After rising to detectable levels, IgM production appears to continue over the course of infection. Average times for antibody detection were also estimated mathematically in a Litchfield-Wilcoxon test (112) (Table 6.1). This method
estimated that 50% of the armadillos would have detectable IgM antibody to Phen-Gl-1 by 186 days post infection with M. leprae. The 95% confidence interval on this 50% day found its boundaries at 150 and 230 days post infection. At 76 days post infection 16% of the animals probably have detectable antibody and at 455 days post infection at least 84% of the armadillos should be positive by the ELISA. In comparison, the average (arithmetic mean) day armadillos might exhibit a disseminated acid-fast bacterial infection as detectable by histologic examination of ear tissues was found to be 519 days post infection. The confidence interval on this average histologic positive day exceeded the boundaries of the plot. Additionally, data from 70 armadillos was sufficient to allow comparison of the ELISA with histologic methods for early identification of positive animals. Using those same 82 day intervals, a positive ELISA preceded a positive histologic examination by at least 82 days 92% (65/70) of the time. ELISA and histology tied 4% (3/70) of the time, and histology was positive before ELISA 2.8% (2/70) of the time.

DISCUSSION

The nine-banded armadillo, Dasypus novemcinctus, is well developed as a host for the in vivo propagation of M. leprae (94,97). Following experimental infection, the armadillo liver commonly yields $10^9$ harvestable M. leprae
per gram of tissue. Unfortunately, not all armadillos succumb to a lepromatous infection and approximately 10% of the animals are resistant to \textit{M. leprae} (95,96,172). Early identification of resistant armadillos would allow their removal from production and aid in identifying animals with unsuitable immunologic characteristics for other studies. These data indicate that the ELISA-IgM v. Phen-Gl-1 is an effective method for monitoring armadillos experimentally infected with \textit{M. leprae}. Serologic monitoring is less labor intensive and may be used effectively earlier in the course of experimental infection by \textit{M. leprae} than histologic methods previously applied.

Histologic examination of ear clip tissues and microscopic examination of buffy coat blood smears for acid-fast bacilli are the only previous objective methods to monitor experimental infections of \textit{M. leprae} in armadillos (97,215). Histologic methods are more laborious than serologic methods and require greater skill and training. When acid-fast bacilli are detected by histologic methods, they indicate a disseminated \textit{M. leprae} infection. Unfortunately, not all armadillos that succumb to \textit{M. leprae} evidence a disseminated infection in ear tissues or blood smears. However, apparently all armadillos that succumb to \textit{M. leprae} produce detectable IgM antibodies to the Phen-Gl-1 antigen and 96% (28/29) have ELISA absorbances greater than 1.20. The average time for histologic methods to indicate a successful disseminated
infection was 519 days. The average time for the ELISA to
detect antibodies was 186 days post-infection. The ELISA
preceded or tied a positive histologic finding by at least
82 days 97% (68/70) of the time. Therefore, in the course
of an experimental infection with \textit{M. leprae}, ELISA
monitoring for IgM antibodies to the Phen-G1-1 antigen
appears to be more predictive, less labor intensive, and an
earlier objective monitoring method than currently used
histologic means.

Resistance to leprosy is governed by cell mediated
immunity \textsuperscript{(86,94)}. Limited observations of resistant
armadillos suggest their IgM antibody production to
Phen-G1-1 is irregular or absent. In successful infections
this antibody production appears to be continuous and
results in high ELISA absorbances at sacrifice. After 455
days, at least 84% of all armadillos should develop
detectable IgM antibodies to the Phen-G1-1 antigen.
Therefore, monitoring armadillos by ELISA may prove useful
in predicting resistance to \textit{M. leprae} early in the course
of experimental infections in armadillos.

Armadillos are known to harbor a naturally acquired
mycobacterial infection that is indistinguishable from \textit{M.
leprae} \textsuperscript{(14,184,185,210,211)}. For the armadillo to be used
effectively as an animal model in leprosy research or in the \textit{in vivo}
propagation of \textit{M. leprae}, care must be taken to
avoid animals experienced with leprosy in the wild.
Armadillos presenting from the wild with detectable
antibodies to Phen-Gl-1 often responded differently from other animals to experimental infection. Early dissemination and progressive rise in antibodies suggests exacerbation of pre-existing infections. A drop in antibody may reflect an unusual immune potential. Therefore, pre-screening armadillos by ELISA might be helpful in avoiding wild-type contamination of culture strains and aid researchers in selecting animals appropriate for study.

The ELISA-IgM v. Phen-Gl-1 appears to have good application as an aid in the management of experimental infections with M. leprae in armadillos. The assay can assist in identifying satisfactory harvest times, resistant animals, and those that experienced leprosy in the wild. Like humans armadillos appear to be somewhat heterogenous in their immunologic responses to M. leprae, but on average appear to develop detectable IgM antibodies to the Phen-Gl-1 antigen approximately 6 months after experimental intravenous inoculum of $5 \times 10^8$ organisms. This lag in IgM antibody response is a curious event and may relate to the size of inoculum or the lipid nature of this antigen. Future studies with this antigen will better refine this ELISA as an aid for the in vivo propagation of M. leprae and may lead to a better understanding of the immune response in leprosy.
Acknowledgements

We express our appreciation to Dr. Darrel Gwinn, National Institutes for Allergy and Infectious Diseases for armadillo plasma samples used in this study, to the staff of the Microbiology Research Department at the National Hansen's Disease Center for armadillo plasma samples and demographic information, to M.J. Morales for computer and statistical consultation, Renee Painter for outstanding secretarial assistance, and S. Christy for technical assistance. This project has been supported in part by a grant from the American Leprosy Missions and a CO-STEP training award from the United States Public Health Service.
Table 6.1

LITCHFIELD-WILCOXON

To determine mean time of IgM antibody positive

<table>
<thead>
<tr>
<th>Days</th>
<th>positive/negative</th>
<th>percent positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>82</td>
<td>3 / 14</td>
<td>21 %</td>
</tr>
<tr>
<td>164</td>
<td>18 / 51</td>
<td>35 %</td>
</tr>
<tr>
<td>246</td>
<td>19 / 34</td>
<td>55 %</td>
</tr>
<tr>
<td>328</td>
<td>20 / 26</td>
<td>76 %</td>
</tr>
<tr>
<td>365</td>
<td>11 / 12</td>
<td>91 %</td>
</tr>
<tr>
<td>492</td>
<td>11 / 15</td>
<td>73 %</td>
</tr>
<tr>
<td>614</td>
<td>2 / 2</td>
<td>100 %</td>
</tr>
<tr>
<td>696</td>
<td>8 / 9</td>
<td>88 %</td>
</tr>
</tbody>
</table>

Table value = 12.952  Chi squared = 7.83

Data are not heterogenous----Curve is a good fit

*PD_{16} = 76 days  PD_{84} = 455 days

PD_{50} = 186 days

(95% confidence interval for PD_{50} 154 to 230 days)

*PD=positive day
Figure 6.1  Yield of *M. leprae* from armadillo livers is compared with ELISA absorbance for IgM antibodies to the Phen-Gl-1 antigen in sacrifice sera. Armadillos yielding $10^7$ *M. leprae* or less per gram of liver tissue are considered to yield $10^7$ bacilli.

Figure 6.2  ELISA absorbances of serial samples from resistant armadillos are related to their elapsed time from inoculation. Detectable antibody ELISA absorbance determined previously must exceed 710 on graph. Resistant armadillos do not appear to produce detectable IgM antibody to Phen-Gl-1 or production is irregular.

Figure 6.3  ELISA absorbances of serial samples from armadillos presenting from the wild with detectable antibodies to Phen-Gl-1 are compared to their histologic status before and after experimental infection with *M. leprae*. Horizontal dotted lines contain equivocal zone for interpretation of ELISA results and were derived previously.
Absorbances below the lines indicate no significant antibody, those above the line indicate antibody present.

Figure 6.4 Mean ELISA absorbances for IgM antibodies to Phen-Gl-1 of 404 armadillo plasma samples are compared over 82 day intervals. Mean absorbance of each interval is plotted at the end of that interval or the last occurring day in the interval and bracketed by the SEM. Horizontal dotted lines (●●●) demark equivocal zone. Absorbances above this zone are considered positive and those below negative. Vertical stipple line (-----) marks 50% positive day estimated by Litchfield-Wilcoxon and vertical dotted lines mark 95% confidence interval on the 50% day. Asterisks (**) bound the 16% and 84% positive day by the same method. Vertical broken line (-----) marks arithmetic mean day for detection of acid-fast bacteria in ear tissues by histologic methods. Figure demonstrates IgM antibodies become detectable in armadillos 186 days after experimental infection and continue to be detectable thereafter.
Figure 6.1 Regression of ELISA Absorbance at Sacrifice vs. Liver Yield of M. leprae
Figure 6.2 ELISA Profile Resistant Armadillos
Figure 6.3 ELISA Profile
Wild Infected Armadillos
Figure 6.4  Kinetics of IgM Antibody to Phen-G1-1
CHAPTER 7

ANTIBODY RESPONSES OF LEPROSY PATIENTS
CONTACTS AND NORMAL CONTROLS
TO THE PHENOLIC-GLYCOLIPID-1
ANTIGEN OF M. LEPRAE

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Chapter 7, Abstract:

Enzyme-linked immunosorbent assays (ELISA) have been developed to detect antibodies to the phenolic-glycolipid-1 (Phen-Gl-1) antigen of *M. leprae*. Statistical definitions for positive/negative interpretations in ELISA were derived by testing sera from 226 leprosy patients, 100 National Hansen's Disease Center (NHDC) staff members and 108 Pima Indians. Leprosy has not been diagnosed in the native American Indian and Pimas reside in a section of the United States where contact with leprosy would be rare. False positive reactions were not found in sera from Pima Indians. Therefore, Pimas probably represent "true negatives" for leprosy serology. IgM, IgA, and IgG antibodies to Phen-Gl-1 were detected in 46-62% of the leprosy patients tested. These antibodies were also detected in 12-14% of presumed healthy NHDC staff members. The predictive value of antibodies to Phen-Gl-1 is unknown. No significant correlation between ELISA absorbances and the bacterial index of lepromatous patients were found. Coefficients of correlation did not exceed 0.08. It is presumed that Phen-Gl-1 may persist in tissues to promote a humoral response even in absence of detectable bacilli. Anti-microbial therapy with dapsone had no significant effect on the serologic profiles of lepromatous patients with respect to serum IgM, IgA, IgG, C3 and specific IgM, IgA, IgG antibodies to the Phen-Gl-1 antigen. Patients suffering erythema nodosum leprosm (ENL) had no discernable
trends in antibodies to Phen-Gl-1. ENL was noted in patients both with and without detectable antibodies to Phen-Gl-1. Serological monitoring of patients to predict ENL does not seem feasible. The drug thalidomide had significant effect on levels of total serum IgM and specific IgM antibodies to Phen-Gl-1. Levels of other serum constituents or specific antibodies to Phen-Gl-1 were not significantly affected. Further studies on the persisting nature of this antigen and its possibility to stimulate a chronic antibody response may yield insight into the character of immune regulation in leprosy.
Descriptions of antibody responses to *M. leprae* have always been complicated by a lack of specificity in antigen systems (61,100). The bacilli bear a number of antigens on their cell surface which cross-react with other mycobacterial species (94,136). Recently the phenolic-glycolipid-1 (Phen-Gl-1) antigen has been isolated from the tissues of armadillos experimentally infected with *M. leprae*. This antigen is chemically defined and apparently species specific for *M. leprae* (22,33,78). The specificity of this antigen makes it useful for sero-epidemiologic studies (24) and provides a unique opportunity to observe *M. leprae* specific immune responses in the human host.

We present here an evaluation of the antibody responses to the phenolic-glycolipid-1 (Phen-Gl-1) antigen of *M. leprae* in leprosy patients, health care personnel in leprosy, and individuals of a leprosy virgin population.

**MATERIALS AND METHODS**

**Serum Samples:** A total of 435 serum samples were obtained for testing by enzyme-linked immunosorbent assay (ELISA) for antibodies to the phenolic-glycolipid-1 antigen of *M. leprae* - 226 leprosy patients at the National Hansen's Disease Center (NHDC) in Carville, Louisiana, 100 presumed healthy NHDC staff members, and 108 Pima Indians from Phoenix, Arizona. Leprosy patients were classified according to the Ridley-Jopling scale (153) and presented
to us as 196 lepromatous (LL), 6 borderline-lepromatous (BL), 14 mid-borderline (BB), and 11 tuberculoid (TT) patients. Leprosy patients were sampled either routinely or purposefully as they presented as new patients or were reactional. Relevant patient information collected included bacterial index; age; sex; reactional status; concentrations of total serum IgM, IgA, IgG and the C3 protein of complement; treatment at the time of sampling according to 17 different regimens with 10 different drugs; as well as ELISA absorbancies for specific IgM, IgA and IgG antibodies to the phenolic-glycolipid-1 antigen.

Bacteriological Index was calculated from the mean of 6 skin scrapings according to the method of Ridley (156). Total human serum IgM, IgA, IgG and the C3 protein of complement were quantitated by radial-immunodiffusion and reported in international units according to the recommendations of the manufacturer (Behring Diagnostics, Summerville, NJ). Drugs were used in standard therapeutic doses and considered here only as "in use" or not.

Sera from 100 NHDC staff members were taken routinely as part of yearly physical examinations. Samples were collected from individuals with no overt signs of leprosy and without regard for race, age, sex, or duties at the NHDC. Identifiers were removed from the samples by clinical laboratory staff and provided as left over diagnostic specimens.
Sera from Pima Indians were collected by Dr. William Knowler, Chief, National Institutes of Health Field Investigations Unit, Phoenix, Arizona, and made available for this study. Sera had been taken as part of a program for diabetes screening and were stored frozen until use. The 108 samples represented Pima Indians of mixed age and sex, who had no overt signs of leprosy and were not undergoing immunosuppressive therapy.

**Antigen**: Native purified phenolic-glycolipid-1 (Phen-Gl-1) was prepared by Dr. Patrick Brennan (Colorado State University) and obtained through contract with the National Institute of Allergy and Infectious Diseases (Dr. Darrel Gwinn, Project Officer).

**ELISA**: Enzyme-linked immunosorbent assay (ELISA) was performed by a modification of the method previously described (198). Specifically, native purified phenolic-glycolipid-1 was mixed with 0.05 M carbonate buffer pH 9.2 and suspended via sonication with a 3 mm probe at 70 watts for 3 minutes (Ultrasonics, Inc., Plainville, NY). The suspended antigen was diluted in the same buffer to a concentration of 2 μg in 50 μl, the volume which was added to 48 wells of a 96 well polyvinyl flatbottom microtiter plate (Cook Labs, Alexandria, VA). The remaining 48 wells received 50 μl of the carbonate buffer without antigen. The plates were sealed with self-sticking cellophane tape and incubated overnight at 4°C. After incubation, the wells were washed 3 times for 7 minutes each wash with 200
μl of wash buffer consisting of 0.067 M phosphate buffered saline (PBS) pH 7.2 containing 1% bovine serum albumin (BSA) (Fraction V, Sigma, St. Louis, MO). After washing, the wells were blocked by the addition of 100 μl of 0.067 M PBS pH 7.2 containing 5% BSA and incubated at 37°C for 1 hour in a water bath. The wells were then washed as before and 50 μl of each serum sample optimally diluted in wash buffer was added to quadruplicate wells of the microtiter plate: 2 with antigen and 2 not containing antigen. The plates were again sealed and incubated at 37°C for 45 minutes when they were removed and washed as before. Horseradish peroxidase conjugated IgG fraction of goat anti-human IgM (μ chain specific), anti-human serum IgA (α chain specific), anti-human IgG (γ chain specific) (Cappel Laboratories, Cochranville, PA) as appropriate and optimally diluted and wash buffer was added to each well in 50 μl volumes. The plates were again sealed and incubated for 45 minutes at 37°C in a water bath when they were removed and washed as before. After washing, 50 μl of a solution containing 0.4 mg/ml ortho-phenylene-diamine (Sigma, St. Louis, MO) with 0.002% H₂O₂ in 0.1 M sodium acetate buffer pH 5.5 was added to each well. The plates were incubated at room temperature for 15 minutes when the reaction was stopped with the addition of 50 μl 9 N HCl. The plates were read for absorbance at 492 nm on a Titertek Multiscan photometer (Flow Labs, Richmond, VA). Mean absorbancies and coefficient of variation (CV)
for each set (pair) of like wells were calculated. If the coefficient of variation exceeded 21%, values were not taken and tests were repeated. Mean absorptions from 8 wells receiving only conjugate and antigen were calculated and subtracted from the mean absorptions of paired antigen coated wells receiving test serum and conjugate. Likewise, the mean absorptions from 8 wells not coated with antigen and receiving only conjugate were subtracted from the mean absorptions of paired wells receiving test serum, conjugate and containing antigen. Finally, the resulting absorbances from test wells without antigen were subtracted from those of test wells with antigen. A quadruplicate series of 8 two-fold dilutions for a high positive lepromatous serum was included in each test run to allow for correction from run to run by the method of least squares. Each serum was tested in at least 2 replicates and their average was used for analysis.

Optimal dilutions for the ELISA were determined as follows: The optimal dilution for enzyme-antibody conjugates used in these ELISA assays was defined to be that dilution of conjugate resulting in the best correlated linear slope of absorbancies when reacted with serial dilutions between 0.1 ng and 10 ng of chromatographically purified human IgM, IgA or IgG as appropriate. For conjugate lots used in this study those effective optimal dilutions were anti-human IgM 1:3200, anti-human IgA
1:12,000, and anti-human IgG 1:24,000. The optimal dilution of test serum was found by checkerboard titration (79). Serial dilutions of a battery of high positive and negative sera were reacted in microtiter wells containing 2 μg Phen-Gl-1 per well and detected by the optimally diluted conjugate. Optimal dilution for the serum was judged to be that dilution which resulted in the greatest difference in absorbancies between the positive and negative sera. In all cases for use in this study, that dilution was found to be 1:35 in the tube.

**Statistical Analysis:** Analysis of data were performed on an IBM 5320 using standard statistical programs in the Statistical Analysis Systems (SAS) commercial program package (36,192).

**RESULTS**

**ELISA-IgM; Evaluation of Positive and Negative Reactions:** Sera from 226 leprosy patients, 100 National Hansen's Disease Center (NHDC) staff members, and 108 Pima Indians were screened by ELISA for IgM antibodies to the phenolic-glycolipid-1 antigen. The resulting absorbancies were pooled and examined in a frequency histogram (Fig. 7.1). Inspection of the histogram showed the population to be positively skewed and a Pearson's coefficient of skewness was calculated to be +1.4. Non-specific reaction in ELISA would be expected to occur
randomly over a normal distribution. Reactions of specific antibodies with the phenolic-glycolipid-1 antigen in ELISA would not be random and they would be expected to deviate from normal. The positive skew of the sample population indicated that the ELISA detected specific antibodies to the Phen-Gl-1 antigen.

To determine if the ELISA described differences between the patient, staff, and Pima groups, their respective absorbances were examined in a scattergram (Fig. 7.2). Inspection of the scattergram suggested that the groups were different. Analysis of variance confirmed that patients were significantly different \((p < 0.001)\) from staff and Pimas while staff and Pimas were also significantly different \((p < 0.05)\). A test for extreme values revealed significant outliers in the staff group but did not detect outliers in the Pima group. Distributions of the individual groups were also examined. Patients and staff were both positively skewed with a Pearson's coefficient of skewness: 1.1 patient and 1.4 staff. The Pima group was more normally distributed having a Pearson's coefficient of skewness of only 0.18.

The approximately normal distribution of ELISA absorbancies from the Pima group suggested random, non-specific detection of IgM antibodies. The positively skewed distribution of patients and staff probably reflected reaction of specific IgM antibodies to the Phen-Gl-1 antigen. Thus, a 99% confidence interval around
the distribution of the Pima absorbancies describes the interval of non-specific detection in the ELISA-IgM, and can be used for positive and negative interpretations. By Z-transformation, the upper boundary of this interval was calculated to be 0.346. Absorbances below this upper limit are within the confidence of non-specific reaction and may be called negative. Absorbancies greater than this upper limit exceed the confidence of non-specific reaction and may be called positive (as they reflect specific reaction of IgM antibodies to the Phen-Gl-1 antigen). Using these definitions, 0% (0/108) Pima Indians were positive for IgM antibodies to the Phen-Gl-1 antigen. However, 12% (12/100) NHDC staff members and 62% (142/226) leprosy patients were positive for IgM antibodies to the Phen-Gl-1 antigen of M. leprae.

**ELISA-IgA; Evaluation of Positive and Negative Reactions:** The combined sample population was also tested by ELISA for IgA antibodies to the Phen-Gl-1 antigen. As described above, the resulting absorbances were inspected in a frequency histogram (Fig. 7.3). Inspection of the frequency histogram suggested a definite positive skew and a Pearson's coefficient of skewness was calculated to be 1.6. A positive skew suggested that the ELISA detected specific IgA antibodies to the phenolic-glycolipid-1 antigen. Closer scrutiny of these groups in a scattergram (Fig. 7.4) suggested that they were different. Analysis of variance described that patients were
significantly different from both staff and Pima's \( (p < 0.001) \) while staff and Pimas were likewise significantly different \( (p < 0.01) \). A test for extreme values found significant outliers in the patient and staff groups but did not identify significant outliers in the Pima group. Additionally, the distributions of the patient and staff groups showed positive skew (Pearson's coefficient of skewness: patients 0.99, staff 3.19). The distribution of the Pima group was more normal having a Pearson's coefficient of skewness of only 0.002. The approximately normal distribution reflected the detection of only non-specific antibodies while the positive skew of patients and staff suggested detection of specific antibodies to the Phen-Gl-1 antigen. Therefore, a 99% confidence interval constructed about the distribution of the Pima group would describe the interval of non-specific reaction in the ELISA and the boundaries of this interval could be used for positive and negative interpretations. Using Z-transformation, the upper boundary of the 99% confidence interval about the Pima group was found to be 0.274. All \( (108/108) \) ELISA absorbancies from the Pima group were found in this interval and the Pimas were interpretable as negative. However, 13% \( (13/100) \) NHDC staff member absorbancies and 46% \( (106/226) \) leprosy patient absorbancies were found outside this interval suggesting specific reaction and detection of specific IgA antibodies to the Phen-Gl-1 antigen.
ELISA IgG: Evaluation of Positive and Negative Reactions: Likewise, IgG antibodies of the combined sample population presented a positive skew of 1.23 in the frequency histogram and scattergram (Fig. 7.5 and 7.6). Analysis of variance indicated there were significant differences in the sample population and showed patients were significantly different from both staff and Pimas ($p < 0.001$), while again the Pima group was significantly different from staff ($p < 0.01$). Significant outliers were not identified in the Pima group but were found in the patient and staff groups. The distribution of the Pima group had a skew of only 0.01; but, the staff and patients were positively skewed with a Pearson's coefficient of 1.22 and 0.827 respectively. Thus, Pimas did not reflect any disturbance in their distribution caused by specific reaction of antibodies to the Phen-Gl-1 antigen; while patients and staff demonstrated a positive skew, probably as the result of specific reaction of antibodies with the phenolic-glycolipid-1 antigen. A 99% confidence interval about the distribution of the Pima's describes the interval of non-specific reaction in the ELISA-IgG and the upper limit of that interval could be used for interpretation of positive and negative reactions. Using $z$-transformation, that upper limit was found to be 0.362. Using these definitions, 100% (108/108) Pima Indians had ELISA absorbancies interpretable as negative. However, 14% (14/100) NHDC staff members and 47% (108/226) leprosy
patients had ELISA absorbancies interpretable as positive for IgG antibodies to the Phen-Gl-1 antigen of *M. leprae*.

**Antibodies by Disease Classification:** The patient group was sorted according to their respective disease classification as lepromatous (LL), borderline-lepromatous (BL), mid-borderline (BB), and tuberculoid (TT) (153). No borderline-tuberculoid patients were sampled. ELISA absorbancies for IgM, IgA, and IgG antibodies to the Phen-Gl-1 antigen resulting from patient sera of these 4 classes are presented in a scattergram (Figs. 7.7, 7.8, 7.9 respectively).

Lepromatous patients dominated the sample and appeared to exhibit the highest ELISA absorbancies for antibodies to the Phen-Gl-1 antigen. The level of ELISA absorbancies decreased when the disease classification became tuberculoid (TT). Antibodies of all classes (IgM, IgA, and IgG) were detected in lepromatous (LL), borderline-lepromatous (BL), and mid-borderline (BB) patients. A single serum sample from a tuberculoid patient had ELISA absorbancies interpretable as positive for IgG antibodies to the Phen-Gl-1 antigen. Neither IgM nor IgA antibodies were detectable in the sera from tuberculoid (TT) patients.

The patients under study included both active and inactive cases on various regimens of treatment. Serum samples were taken at a single point in time and longitudinal screening was not sought. On a percentage basis, 63% (123/196) of the lepromatous (LL) patients, 100%
(6/6) of the borderline-lepromatous (BL) patients, and 28% (4/14) of the mid-borderline (BB) patients had detectable IgM antibodies to the phenolic-glycolipid-1 antigen. Likewise, IgA antibodies were detected in 47% (92/196) of the lepromatous (LL) patients, 66% (4/6) of the borderline-lepromatous (BL) patients, and 14% (2/14) of the mid-borderline (BB) patients; while IgG antibodies were detected in 48% (93/196) of the lepromatous (LL) patients, 50% (3/6) of the borderline-lepromatous (BL) patients, 29% (4/14) of the mid-borderline (BB) patients, and 9% (1/11) of the tuberculoid (TT) patients. More than 1 antibody isotype was detected in 32% (72/226) of the patient sera, and all 3 antibody isotypes were detected in 18% (40/226) of the patient sera tested.

There were no significant trends with regard to detection of antibody isotypes in combination. While IgM antibody was detected more frequently, it could equally well be found independently or in combination with IgA or IgG. Likewise, IgA and IgG showed no significant trend with regard to detection in combination.

**Correlation of ELISA with Bacterial Index (BI):**

ELISA absorbancies of IgM, IgA and IgG antibodies to the Phen-Gl-1 antigen and the bacterial index (BI) were compared by regression. Previous comparisons had shown that serologic profiles of patients treated only with dapsone or rifampin or on combined therapy of dapsone and rifampin were not significantly different. For comparison
of ELISA absorbances according to BI only polar lepromatous patients treated with dapsone or dapsone in combination with rifampin, were compared (Figs. 7.10, 7.11, 7.12). No significant correlations between ELISA absorbancies and BI was found. Correlation coefficients \( r \) did not exceed 0.08. Slopes of the regression lines for ELISA absorbancies of IgM, IgA and IgG antibodies to the phenolic-glycolipid-1 antigen with respect to BI were not significant. The slope of the IgG regression line was negative. The 95% confidence intervals about the regression lines were wide and disparate. The 95% confidence interval for ELISA-IgM absorbancies with respect to BI exceeded the boundaries of the plot.

Levels of Phen-Gl-1 Antibodies with Treatment:
ELISA absorbancies for specific antibodies to the Phen-Gl-1 antigen were compared for lepromatous patients undergoing different therapeutic regimens. Concentrations of total serum IgM, IgA, IgG and the C3 protein of complement were also compared. Concentrations of total serum immunoglobulins and C3 were not available for all patients. Sample sizes of the groups were also different and the varying degrees of freedom (df) are recorded in the appropriate tables. Significant differences between the groups were described by analysis of variance and a Bonferroni (Dunn) \( t \)-test. Statistical comparisons using these methods are quite conservative and the Bonferroni
t-test controls the type I experimental error but generally has the higher type II error rate than Tukey's (36,192).

The selected groups were evaluated for differences between treatment, sex, and treatment-sex interactions. Significant differences influenced by sex were not observed in ELISA absorbancies for antibodies to the phenolic-glycolipid-1 antigen, nor in concentrations of total immunoglobulins. Significant differences influenced by sex were observed in C3 concentrations. When sex had a significant influence on C3 but treatment did not, those differences were reported here as not significant. No significant differences in C3 concentrations are observed in the treatments examined.

Patients untreated for active leprosy and patients on dapsone anti-microbial therapy for leprosy are compared in Table 7.1. No significant differences were observed between dapsone treated patients and untreated patients.

Patients continuing on dapsone therapy but undergoing a reactional episode of erythema nodosum leprosum (ENL) are compared to non-reactional dapsone treated patients in Table 7.2. Total serum IgM antibodies were significantly higher in the reactional patient. Total serum IgA, IgG, C3 and specific antibodies to the Phen-Gl-1 antigen were not observed to be significantly different in the dapsone treated patient and reactional patient on the same therapy. Patients with ENL had ELISA-IgM, IgA, and IgG absorbancies ranging from positive to undetectable in the ELISA.
Patients on dapsone therapy and undergoing ENL are compared to patients on therapy with a combination of dapsone and thalidomide (the latter as part of treatment for ENL) in Table 7.3. Patients treated with thalidomide have significantly lower levels of total serum IgM than patients in ENL and not taking thalidomide. Total serum IgA, IgG and C3 were not significantly different. ELISA absorbancies for IgM antibodies to the Phen-Gl-1 antigen were also significantly lower in the thalidomide treated patient. Specific IgA and IgG antibodies were not significantly affected by treatment with thalidomide.

Patients on dapsone therapy but not in ENL were compared to patients on combined therapy of dapsone and thalidomide in Table 7.4. The dapsone-thalidomide treated patient had significantly lower total serum IgM and ELISA-IgM for antibodies to the Phen-Gl-1 antigen than patients on dapsone only. Concentrations for total serum IgA, IgG and C3, as well as specific IgA and IgG ELISA absorbancies, were not shown to be significantly different.

DISCUSSION

Using ELISA, IgM, IgA and IgG antibodies to the phenolic-glycolipid-1 antigen of \textit{M. leprae} were detectable in the sera of leprosy patients. These antibodies were also detectable in sera from staff members at the National Hansen's Disease Center (NHDC), but were not detectable in
sera from Pima Indians. Leprosy has not been diagnosed in the native American Indian and Pima's reside in a section of the United States where reports of leprosy are extremely rare (58,68). In that manner, Pima Indians might be considered as leprosy virgin and sera from this group may be used as negative controls in leprosy serology.

ELISA yield results in objectively derived numeric absorbance units that are readily used in statistical analysis. Using ELISA absorbancies for sera from Pima Indians, statistical definitions for interpretation of positive/negative ELISA results were derived. Pima Indian sera were shown to react non-specifically over an approximately normal distribution in ELISA for antibodies to the Phen-Gl-1 antigen. ELISA absorbancies for sera from leprosy patients and NHDC staff did not result in normal distributions but showed positive skew. This skew reflected reaction of specific antibodies with the Phen-Gl-1 antigen. A 99% confidence interval constructed about the Pima distribution described the range for non-specific reactions in the ELISA. The upper boundary of that interval described the probable upper limit for non-specific reaction and was used in definition of positive and negative in ELISA absorbancies.

Using these definitions, antibodies to the Phen-Gl-1 antigen were detected in 12-14% of presumed healthy NHDC staff members. Antibody prevalence rates have not previously been reported for health care delivery personnel
in leprosy. Antibodies to the phenolic-glycolipid-1 antigen have been reported in family members and contacts of leprosy patients (24,217). Some NHDC staff members come in constant contact with leprosy patients and are likely hyper-exposed to *M. leprae* in their environment. Leprosy personnel have been noted to have higher relative risk rates for contracting leprosy than other groups (51). These data indicate that staff members also have a high probability of developing antibodies to *M. leprae*. At this time, the relative risk, as may be ascribed to persons with a known environmental exposure to *M. leprae* and having positive serological indications, cannot be discerned. The predictive value of IgM antibodies to Phen-Gl-1 is a continuing research interest for sero-epidemiology.

Leprosy patients may be classified according to the 5 group scheme of the Ridley-Jopling scale (153). This method groups patients according to their own disease status and recognizes the split anergy of the leprosy immune response (26,31,101). Polar lepromatous patients have a predominant humoral immune response but lack competent cell mediated immunity to *M. leprae*. Patients to the tuberculoid pole have a predominant cell-mediated immune response but lack strong humoral immunity to *M. leprae*. Patients in this study also seemed to reflect that dichotomy with regard to IgM, IgA and IgG antibodies to the Phen-Gl-1 antigen. These observations are in keeping with those of previous investigators who noted a decline in IgM
or total immunoglobulins as patient classification moved from polar lepromatous (LL) to polar tuberculoid (TT) (19,26,133). ELISA-IgM absorbancies in lepromatous patients also were the highest recorded. IgM was the most commonly detected isotype in leprosy patients. These data are in keeping with assertions by previous investigators that IgM antibodies to Phen-Gl-1 may be the most appropriate isotype for sero-epidemiology (24,217). However, IgM, IgA and IgG antibodies to the Phen-Gl-1 antigen were also detectable in persons environmentally exposed to M. leprae and in treated and untreated leprosy patients. Though no discernable trends could thus far be revealed in detecting combinations of antibodies, isotypes other than IgM may have relevant roles in sero-epidemiology and clinical investigations.

To determine if levels of Phen-Gl-1 antibodies in lepromatous patients might be affected by their status at a given point in time, selected groups were examined. Using extracts of M. leprae, previous investigators have asserted correlation between the level of antibody activity and the bacterial load of the lepromatous patient (130,131). ELISA absorbancies for IgM, IgA, and IgG antibodies to the phenolic-glycolipid-1 antigen had no significant correlations with the bacterial index of lepromatous patients on anti-microbial therapy. As shown in Figures 7.9, 7.10, and 7.11, even lepromatous patients with a BI of 0 may have detectable antibodies to the Phen-Gl-1 antigen.
The persistence of detectable antibody in patients with a BI of 0 probably indicates that this antigen persists after acid-fast bacilli have been cleared. Granulomas of resolving lepromatous patients contain no detectable bacilli but foamy macrophages may be detected (85). Foamy inclusions in these macrophages may be lipid from *M. leprae* and could be responsible for the persisting humoral response.

To determine if serologic profiles of lepromatous patients were influenced by drug therapy or erythema nodosum leprosum (ENL), selected groups were examined. Concentrations of total serum immunoglobulins, the C3 protein of complement as well as ELISA absorbancies for IgM, IgA and IgG antibodies to the Phen-Gl-1 antigen, were tested for significant differences. The groups were selected to assess 4 phases of the disease in the lepromatous patient: untreated new patient, antimicrobially treated, reactional, and treated for reaction.

When an untreated leprosy patient first presents, he is started on a course of anti-microbial therapy. Dapsone is an anti-microbial in common use for the treatment of leprosy. In this study, patients on anti-microbial therapy with dapsone had a similar serologic profile to untreated patients. Previous reports have suggested that anti-microbial therapy over time tends to decrease levels of IgM antibodies (131,132,135). These reports were based on longitudinal sampling of individual patients.
Longitudinal samples were not tested in this study and individual patients were noted to be highly heterogeneous. Previous studies have also noted great heterogeneity in antibody responses from leprosy patients of similar groups (130,133). Therefore, the observations reported here do not contradict those previous conclusions.

Patients undergoing an episode of erythema nodosum leprosum (ENL) had significantly elevated levels of total serum IgM over non-reactional dapsone treated patients. ENL is thought to be an Arthus type hypersensitivity reaction possibly mediated by IgM antibodies (29,87,178, 212,214). Previous investigators have noted elevated serum IgM during the reactional episode and hypothesized that their reaction with M. leprae antigens might initiate ENL (30,170,178). If the Phen-Gl-1 antigen is involved in ENL then one might expect changes in ELISA absorbances of ENL patients depending on whether: a) there was new production of antibody; or b) there was excess consumption of antibody. No changes in ELISA absorbances would be expected if: c) there is a relative balance between production and consumption of Phen-Gl-1 antibodies in ENL; or d) antibodies to Phen-Gl-1 are not involved in ENL. In the present study, ELISA absorbances for specific antibodies to Phen-Gl-1 were not significantly different in reactional patients. Some patients in ENL did not have detectable antibody to Phen-Gl-1. No trends were revealed in detecting antibody that might make serological
monitoring useful in predicting ENL. Therefore, these data provide no evidence that Phen-Gl-1 is directly involved in the pathogenesis of ENL.

On the other hand, patients treated with thalidomide had significantly depressed ELISA-IgM absorbancies for antibodies to the Phen-Gl-1 antigen. Levels of total serum IgM were also significantly lower. Thalidomide has been shown to polyclonally suppress de novo IgM production to T-dependent antigens but not to T-independent antigens (175,176). The suppression seen here in total serum IgM and specific IgM antibodies may be the result of polyclonal suppression or could indicate that IgM antibodies to the phenolic-glycolipid-1 antigen are consumed during ENL. If consumed, their production is inhibited by the action of thalidomide. Therefore suppression of IgM antibodies to the Phen-Gl-1 antigen by the action of thalidomide suggests that Phen-Gl-1 is a T-dependent antigen of \textit{M. leprae}. This is in keeping with the appearance of IgM antibody to Phen-Gl-1 in \textit{M. leprae} infected athymic nude mice only after reconstitution with spleen cells form syngeneic donors (unpublished observations).

In summary, statistical definitions have been developed for the interpretation of positive and negative reactions in ELISA for IgM, IgA, and IgG antibodies to the phenolic-glycolipid-1 antigen of \textit{M. leprae}. Using these definitions, IgM, IgA, and IgG antibodies were detectable in the sera of leprosy patients, and more commonly
detectable in those not classified as tuberculoid. These antibodies were also detectable in presumably healthy individuals at least some of whom are environmentally exposed to *M. lepra*ae. The predictive value of detecting these antibodies in persons environmentally exposed to *M. lepra*ae cannot be ascertained. False positive reactions for antibodies to the Phen-Gl-1 antigen were not found in a group of native American Pima Indians. While not immune to mycobacterial infections, leprosy has never been diagnosed in the native American Indian and Pimas reside in a region of the United States where contact with *M. lepra*ae would be rare. Therefore, Pimas probably represent "true negatives" for leprosy serology. There appear to be no significant correlations between ELISA absorbancies for antibodies to the Phen-Gl-1 antigen and the bacterial index of the lepromatous patient. It is presumed that this lipid antigen may persist in tissues to promote a humoral response even in the absence of detectable acid-fast bacilli. Anti-microbial therapy with dapsone had no detectable effect on the serologic profiles of lepromatous patients. Antibodies to the phenolic-glycolipid-1 antigen could not be implicated directly in the pathogenesis of ENL. There were no discernible trends in ELISA absorbancies for antibodies to this antigen in patients suffering ENL and prospective monitoring of patients by ELISA in hopes of predicting ENL does not seem feasible. The drug thalidomide had significant effect on IgM antibodies to
this antigen and Phen-Gl-1 may be a T-dependent antigen of
M. leprae. Further studies on the persisting nature of
this antigen and its ability to stimulate a chronic
T-dependent response may yield interesting insight into the
character of immune regulation in leprosy.

Acknowledgements

We express our appreciation to Mr. M.J. Morales for
statistical consultation and graphics, and Ms. Renee
Painter for secretarial assistance. This work has been
supported in part by a grant from the Hansen's Disease
Foundation and a CO-STEP training award from the United
States Public Health Service.
Table 7.1 Serologic profile of untreated lepromatous patients and lepromatous patients treated with dapsone

<table>
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<th></th>
<th>DAPSONE</th>
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<td>$\bar{X}$</td>
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<tr>
<td>IgM</td>
<td>197</td>
<td>83</td>
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<tr>
<td>IgA</td>
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<td>102</td>
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<tr>
<td>IgG</td>
<td>188</td>
<td>35</td>
</tr>
<tr>
<td>C3</td>
<td>106</td>
<td>23</td>
</tr>
<tr>
<td>ELISA#</td>
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<td></td>
</tr>
<tr>
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<td>464</td>
</tr>
<tr>
<td>IgA</td>
<td>329</td>
<td>293</td>
</tr>
<tr>
<td>IgG</td>
<td>432</td>
<td>257</td>
</tr>
</tbody>
</table>

$x$=mean; $s$=standard deviation; $df$=degrees freedom; $p$=probability by $t$-test; NS=not significant

*levels are expressed for total serum concentration in IU/dl

#ELISA absorbances for specific antibodies to Phen-Gl-1 antigen.
Table 7.2. Serologic profile of lepromatous patients treated with dapsone but not in reaction and those treated with dapsone but undergoing erythema nodosum leprosum.

<table>
<thead>
<tr>
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<th>p</th>
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<tr>
<td></td>
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<td>$s$</td>
<td>$\bar{X}$</td>
<td>$s$</td>
<td>df</td>
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</tr>
<tr>
<td>Serum* IgM</td>
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<td>83</td>
<td>264</td>
<td>225</td>
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<tr>
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<td>273</td>
<td>147</td>
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<td>NS</td>
</tr>
<tr>
<td>IgG</td>
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<td>35</td>
<td>196</td>
<td>135</td>
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<td>NS</td>
</tr>
<tr>
<td>C3</td>
<td>106</td>
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<td>13</td>
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<td>NS</td>
</tr>
<tr>
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<td>342</td>
<td>33</td>
<td>NS</td>
</tr>
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<td>561</td>
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<tr>
<td>IgG</td>
<td>430</td>
<td>267</td>
<td>361</td>
<td>221</td>
<td>33</td>
<td>NS</td>
</tr>
</tbody>
</table>

$x$=mean; $s$=standard deviation; $df$=degrees freedom; $p$=probability by $t$-test; NS=not significant

*levels are expressed for total serum concentration in IU/dl

#ELISA absorbances for specific antibodies to Phen-Gl-1 antigen.
Table 7.3. Serologic profile of lepromatous patients on dapsone and those on combination therapy of dapsone with thalidomide.

<table>
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<tr>
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<td>( \bar{X} )</td>
<td>S</td>
<td>df</td>
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<tr>
<td>Serum* IgM</td>
<td>197</td>
<td>83</td>
<td>151</td>
<td>62</td>
<td>28</td>
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</tr>
<tr>
<td>IgA</td>
<td>263</td>
<td>102</td>
<td>197</td>
<td>101</td>
<td>28</td>
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<tr>
<td>IgG</td>
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<td>35</td>
<td>169</td>
<td>55</td>
<td>28</td>
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<tr>
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<td>12</td>
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<td>NS</td>
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</tr>
<tr>
<td>ELISA# IgM</td>
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<td>377</td>
<td>328</td>
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<td>0.05</td>
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</tr>
<tr>
<td>IgA</td>
<td>329</td>
<td>293</td>
<td>224</td>
<td>192</td>
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<td>NS</td>
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<tr>
<td>IgG</td>
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<td>523</td>
<td>235</td>
<td>39</td>
<td>NS</td>
<td></td>
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</table>

* X=mean; s=standard deviation; df=degrees freedom; p=probability by t-test; NS=not significant

* Levels are expressed for total serum concentration in IU/dl

# ELISA absorbances for specific antibodies to Phen-G1-1 antigen.
Table 7.4. Serologic profile of lepromatous patients on dapsone in reaction with erythema nodosum leprosum and patients on combination therapy of dapsone with thalidomide

<table>
<thead>
<tr>
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<th>REACTIONAL</th>
<th></th>
<th>THALIDOMIDE</th>
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<td>$\bar{x}$</td>
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<tr>
<td>Serum*</td>
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<td>IgM</td>
<td>264</td>
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<td>IgM</td>
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<td>IgG</td>
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<td>235</td>
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</table>

x=mean; s=standard deviation; df=degrees freedom;
p=probability by $t$-test; NS=not significant
*levels are expressed for total serum concentration in IU/dl
#ELISA absorbances for specific antibodies to Phen-G1-1 antigen.
Table 7.5
Legends to figures.

Figure 7.1 Frequency histogram of ELISA absorbances for IgM antibodies to Phen-Gl-1 from 226 leprosy patients, 100 NHDC staff, and 108 Pima Indians. Absorbance is X 1000 and plot is over 30 equal class intervals of 50 absorbance units each.

Figure 7.2 Scattergram of ELISA absorbances for IgM antibodies to Phen-Gl-1 sorted according to group. Absorbance is coded X 1000. Bars mark the mean of each group. Brackets were found by Z-transformation and indicate the upper 99% confidence limit of each distribution. Horizontal line at 346 demarks limit for non-specific ELISA absorbances as described by Pima distribution.

Figure 7.3 Frequency histogram of ELISA absorbances for IgA antibodies to Phen-Gl-1 from 226 leprosy patients, 100 NHDC staff, and 108 Pima Indians. Absorbance is X 1000 and plot is over 30 equal class intervals of 50 absorbance units each.

Figure 7.4 Scattergram of ELISA absorbances for IgA antibodies to Phen-Gl-1 sorted according to
group. Absorbance is coded X 1000. Bars mark the mean of each group. Brackets were found by Z-transformation and indicate the upper 99% confidence limit of each distribution. Horizontal line at 274 demarks limit for non-specific ELISA absorbances as described by Pima distribution.

Figure 7.5 Frequency histogram of ELISA absorbances for IgG antibodies to Phen-Gl-1 from 226 leprosy patients, 100 NHDC staff, and 108 Pima Indians. Absorbance is X 1000 and plot is over 30 equal class intervals of 50 absorbance units each.

Figure 7.6 Scattergram of ELISA absorbances for IgG antibodies to Phen-Gl-1 sorted according to group. Absorbance is coded X 1000. Bars mark the mean of each group. Brackets were found by Z-transformation and indicate the upper 99% confidence limit of each distribution. Horizontal line at 363 demarks limit for non-specific ELISA absorbances as described by Pima distribution.

Figure 7.7 Scattergram of ELISA absorbances for IgM antibodies to Phen-Gl-1 for 226 leprosy patients sorted according to classification:
LL=lepromatous, BL=borderline lepromatous, BB=mid-borderline, TT=tuberculoid

Figure 7.8 Scattergram of ELISA absorbances for IgA antibodies to Phen-Gl-1 for 226 leprosy patients sorted according to classification. LL=lepromatous, BL=borderline lepromatous, BB=mid-borderline, TT=tuberculoid.

Figure 7.9 Scattergram of ELISA absorbances for IgG antibodies to Phen-Gl-1 for 226 leprosy patients sorted according to classification. LL=lepromatous, BL=borderline lepromatous, BB=mid-borderline, TT=tuberculoid.

Figure 7.10 Correlation by regression of ELISA absorbances for IgM antibodies to Phen-Gl-1 and bacterial index of nonreactional LL patients on therapy with dapsone or dapsone in combination with rifampin. Absorbance is coded X 1000. Correlation coefficient=(r). Broken line (---) is regressed, not significant. Confidence interval of regressed line exceeded the boundaries of the plot.

Figure 7.11 Correlation by regression of ELISA absorbances for IgA antibodies to Phen-Gl-1 and
bacterial index of nonreactional LL patients on therapy with dapsone or dapsone in combination with rifampin. Absorbance is coded X 1000. Correlation coefficient=(r). Broken line (---) is regressed, not significant. Solid line is 95% confidence interval of regression line.

Figure 7.12 Correlation by regression of ELISA absorbances for IgG antibodies to Phen-Gl-1 and bacterial index of nonreactional LL patients on therapy with dapsone or dapsone in combination with rifampin. Absorbance is coded X 1000. Correlation coefficient=(r). Broken line (---) is regressed, not significant. Solid line is 95% confidence interval of regression line.
Figure 7.2 Scattergram ELISA-IgM v. Phen-G1-1
Figure 7.3 Frequency Histogram ELISA-IgG v. Phen-CI-1
Figure 7.4 Scattergram ELISA-IgA v. Phen-Gl-1
Figure 7.5 Frequency Histogram ELISA-IgG v. Phen-G1-1
Figure 7.6 Scattergram ELISA-IgG v. Phen-G1-1
Figure 7.7 Scattergram ELISA-IgM v. Phen-G1-1 by Class
Figure 7.8 Scattergram ELISA-IgA v. Phen-Gl-1 by Class
Figure 7.9 Scattergram ELISA-IgG v. Phen-G1-1 by Class
Figure 7.10 Regression ELISA-IgM with B.I.
Figure 7.11 Regression ELISA-IgA with B.I.
Figure 7.12 Regression ELISA-IgG with B.I.
SUMMARY

In summary, enzyme-linked immunosorbent assays (ELISA) have been developed to detect antibodies to a chemically defined and apparently species specific phenolic-glycolipid-1 (Phen-Gl-1) antigen of *Mycobacterium leprae*. Previous serologic procedures have lacked both sensitivity and specificity as they relied on undefined antigen mixtures and were complicated by the plethora of cross reacting antigenic determinates of mycobacterial species. The assays have been developed with statistical definitions for interpretation of positive and negative reactions. They are developed for application in the two major hosts of leprosy: man and armadillos.

The immunoglobulin IgM is an evolutionarily primitive molecule. Serologic cross reactivities of IgM antibodies have been noted among a number of species. Such cross reactivity was also noted between human and armadillo IgM. Using commercially prepared enzyme-anti-antibody conjugates directed against human IgM, armadillo IgM antibodies are detectable by ELISA. The ELISA-IgM v. Phen-Gl-1 was developed for use in armadillos and evaluation showed it to be highly sensitive and specific. The assay was optimized to have a calculatable specificity of 100%. Such high specificity is especially useful in sero-epidemiologic studies.
Leprosy is zoonotic in the armadillo, *Dasypus novemcinctus*. The origin of these infections has been the subject of great debate. Understanding their origin may have impact on our understanding of the transmission and distribution of leprosy. A retrospective serological survey of armadillos taken in years pre-dating their use in leprosy, found antibodies to the phenolic-glycolipid-1 antigen in 17 of the armadillos examined. These antibodies were not absorbable with other mycobacterial species and presumably were elicited as a result of contact with *M. leprae*. Thus, the disease of armadillos appears to be a naturally acquired zoonosis.

The transmission of leprosy is not yet understood. Studies of the disease are complicated by a long incubation period, low susceptibility, and high mobility of the human population. However, naturally acquired leprosy is zoonotic in the armadillo and may be used as an animal model to study the transmission of leprosy. Transmission may be affected by environmental conditions, population characteristics or other intricacies in the transmission of leprosy. To better develop the armadillo as an animal model for the transmission of leprosy, prevalence rates were estimated in armadillos from two parishes in Louisiana. Modern antibody prevalence rates for Louisiana averaged 12.5%. Variations in prevalence were noted between Louisiana and Florida where there have been no reports of zoonotic leprosy. These variations must be the result of
some factors affecting the transmission of the disease. To better understand these factors, data base information concerning the leprosy positive armadillo habitat of Louisiana has been developed. Future comparison of prevalence in armadillos of similar and different habitats will aid our understanding of factors that influence transmission of leprosy.

*M. leprae* have not been cultivated on artificial media and must be propagated *in vivo*. The armadillo (*Dasypus novemcinctus*) is well developed as a host for *M. leprae*, and is evolving as an animal model for the study of leprosy. Understanding the immunologic responses of armadillos as a result of experimental infection with leprosy will advance their use as models and may impact the efficiency of *in vivo* propagation. Testing of armadillos for IgM antibodies to the phenolic glycolipid-1 antigen showed that there is a slow initial IgM response to this antigen requiring an average of 186 days to become detectable by the ELISA. These antibodies appear to persist over the course of infection and may be detected for up to 1140 days. Armadillos that succumb to *M. leprae* infection develop high antibody levels and ELISA absorbances of greater or less than 1.2 may be used to predict the outcome of an experimental infection 97% of the time. Armadillos that do not succumb to experimental infection appear to have an irregular or absent IgM antibody response to the phenolic-glycolipid-1 antigen. Antibodies were earlier and more
reliable indicators of infection than previously used histologic monitors. These response profiles may be used to predict resistance in armadillos both early and late in the course of experimental infection and will have impact on the efficiency of *in vivo* propagation. Armadillos with naturally acquired leprosy were also noted to have irregular response patterns. Pre-screening of armadillos to identify animals with a wild infection will aid researchers in preserving their culture strains of *M. leprae* and will allow identification of animals that would be unsuitable for modeling studies. Knowledge of the immunologic responses of armadillos to *M. leprae* antigens may benefit our understanding of the immune response of human leprosy patients.

Leprosy has its greatest impact as a severe infection of man. The leprosy patient presents with a complex array of clinical and immunologic symptoms, and management of the disease is complicated. To determine if serologic monitoring may have impact on the diagnosis and management of human leprosy, ELISA were developed. Statistical definitions were developed for the interpretation of positive and negative reactions in ELISA for IgM, IgA, and IgG antibodies to the phenolic glycolipid-1 antigen of *M. leprae*. Using these definitions, IgM, IgA, and IgG antibodies were detectable in the sera of leprosy patients, and more commonly detectable in those not classified as tuberculoid. These antibodies were also detectable in presumably healthy individuals who are
environmentally exposed to *M. leprae*. The predictive value of detecting these antibodies in persons environmentally exposed to *M. leprae* cannot be ascertained. False positive reactions for antibodies to the phenolic-glycolipid-1 antigen were not found in a group of native American Pima Indians. While not immune to mycobacterial infections, leprosy has never been diagnosed in the native American Indian and Pimas reside in a region of the United States where contact with *M. leprae* would be rare. Therefore, Pimas probably represent "true negatives" for leprosy serology. There appear to be no significant correlations between ELISA absorbancies for antibodies to the phenolic-glycolipid-1 antigen and the bacterial index of the lepromatous patient. It is presumed that this lipid antigen may persist in tissues to promote a humoral response even in the absence of detectable acid-fast bacilli. Anti-microbial therapy with dapsone had no significant effect on the serologic profiles of lepromatous patients. Antibodies to the phenolic-glycolipid-1 antigen could not be implicated directly in the pathogenesis of ENL. There were no discernible trends in ELISA absorbancies for antibodies to this antigen in patients suffering ENL and prospective monitoring of patients by ELISA in hopes of predicting ENL does not seem feasible. The drug thalidomide had significant effect on IgM antibodies to this antigen and phenolic-glycolipid-1 may be a T-dependent antigen of *M. leprae*. Further studies on the persisting nature of this antigen and its propensity to stimulate a chronic T-dependent
response, may yield interesting insight into the character of immune regulation in leprosy.
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Richard Truman was born 18 February 1952 in South Charleston, West Virginia. Educated in public schools of Kentucky, North Carolina, Ohio and West Virginia, he completed his secondary education in 1970 at Bluefield High School in Bluefield, West Virginia. He attended Bluefield State College in Bluefield, West Virginia, and received his Bachelorate degree in Biology at West Virginia University in 1974. He was employed as a Research Associate in the Veterinary Science Department at Louisiana State University and received a Masters degree in Microbiology in 1978 from that institution. Following a period of employment and training in public health with the State of Louisiana Department of Health and Human Resources he returned to Louisiana State University and completed his Doctorate in Veterinary Microbiology and Parasitology in 1985. During his studies he was the recipient of the McKlesky Award from the South Central Branch of the American Society for Microbiology; and the J.D. Lane Award in the junior investigator category from the United States Public Health Service Professional Association.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Richard W. Truman

Major Field: Veterinary Microbiology and Parasitology

Title of Dissertation: HOST RESPONSES TO THE PHENOLIC-GLYCOLIPID-1 ANTIGEN OF MYCOBACTERIUM LEPRAE.

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Dean of the Graduate School

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