

Do Antibodies to Phospholipid Antigens Play Any Role in Murine Leprosy?¹

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Human leprosy is typically described as a disease that primarily affects the skin and nerves and secondarily other (internal) organs (¹²). Murine leprosy, to the contrary, is recognized as a disease that primarily affects the viscera and rarely the skin of rats and mice (¹⁶). This later description is probably inexact since for many years the disease has been studied in an experimental manner using the non-natural, intraperitoneal or intravenous, routes of infection. Under natural circumstances, transmission of murine leprosy is very likely to occur through the mucosal surfaces or through abrasions in the skin, just as it happens in the human being (¹²). This might lead to clinical signs similar to the ones seen in human leprosy. Notwithstanding, and depending on the host strain (most of them do), mice infected intraperitoneally with *Mycobacterium lepraemurium* eventually present obvious skin lesions, with a small percentage of them (1% to 3%) additionally developing bilateral paralysis of the rear limbs as a likely manifestation of nerve involvement (this communication).

Nerve involvement has long been recognized as a characteristic of human leprosy, and it is still one of the two typical features on which a diagnosis can be reliably made clinically and histopathologically (the other one is skin affection). There are several

other diseases in which nerves are affected. Guillain-Barre syndrome (GBS), for instance, is a neurological disorder characterized by an inflammatory demyelination of the peripheral nerves. It has been shown that some GBS patients produce autoantibodies to various phospholipid antigens, which may play a role in the myelin damage (⁹). Antiphospholipid antibodies are also common in systemic lupus erythematosus (SLE), and their role in the vascular and neural pathology of the disease also has been suggested (¹⁴). In leprosy, some investigators have thought of the possible participation of antiphospholipid antibodies in the neurological alterations of the disease. Antibodies to phospholipids and to certain mycobacterial lipids [such as phenolic glycolipid-I (PGL-I)] have been detected in the serum of people with lepromatous leprosy (^{4, 5, 7, 10, 17}). Most authors, however, have not found any association between the presence of anti-lipid antibodies and the neural pathology of the disease. Some others, on the other hand, have suggested such an association (^{15, 18}). Nerve affection in experimental murine leprosy also has been reported (¹⁹).

Considering the participation of antiphospholipid antibodies in the pathology of murine leprosy as a possibility, in this investigation we have looked for the presence of antibodies to several lipid and phospholipid antigens, including mycobacterial lipids, lipids extracted from the host tissues (brain, liver and spleen), bovine cerebroside sulfatide and cardiolipin in the serum of mice infected with *M. lepraemurium* by the intraperitoneal route. We used this route of infection because a) we do not know the exact natural route of infection; b) it assures infection of over 90% of the animals; and c) the infection evolves to produce a lepromatous-like (lepromatoid) disease that some-

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what resembles lepromatous leprosy in humans.

MATERIALS AND METHODS

Chemicals. Most chemicals were purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A.; exceptions are indicated.

Animals and their infection. Female, albino, 2-month old, NIH mice were infected with 5×10^7 cells of *M. lepraemurium* (Hawaiian strain) by the intraperitoneal route. Bacilli had been freshly isolated from the spleens of heavily infected NIH mice by using the whole procedure of Prabhakaran (¹⁴) and the Percoll step of Draper (⁶) in that order. Six months after inoculation, the mice were sacrificed under chloroform anesthesia and exsanguinated by heart puncture. Blood was used as the source of serum for the search for antibodies to several lipid species. Two mice that had developed paralysis of the rear limbs and 14 animals that had not were included in the study (globally, paralysis affected 1%–3% of the *M. lepraemurium*-infected animals). The Figure shows a palsied mouse.

Sonically extracted mycobacterial antigens. A 20% suspension (v/v) of highly purified bacilli in saline-borate solution (pH 8.4) was subjected to disruption in a sonifier (Sonifier, Danbury, Connecticut, U.S.A.) for 15 min (5×3 -min pulses) at 80 watts. The sonicate was then centrifuged at $15,000 \times g \times 10$ min, the supernatant collected, and 1 mM N-ethyl maleimide and 1 mM p-phenyl sulfonyl fluoride were added. Sterilized through a 0.45- μ m Millipore membrane, the supernatant was divided into 1.0-ml aliquots and kept frozen at -20°C until used.

***M. lepraemurium*-derived lipids.** Mycobacterial lipids were prepared according to Folch, *et al.* (⁸) for whole lipids and Luna, *et al.* (¹¹) for lipids of low, intermediate, and high polarity.

Lipids from normal tissue. Lipids were prepared from the liver and spleen of normal animals according to Folch, *et al.* (⁸). Lipids of the brain were obtained by thoroughly grinding a mouse brain with about 10 ml of absolute ethanol in a glass-tissue homogenizer. After centrifuging the ho-



THE FIGURE. Some NIH mice (1%–3%) infected with *M. lepraemurium* eventually develop bilateral paralysis of the rear limbs. Paralysis appears late in the infection and does not affect the front limbs.

mogenate, the ethanolic extract was collected with no further treatment in a small, wide-mouth, screw-cap flask and the alcohol allowed to evaporate at 37°C . This led to a whitish waxy material that was kept in a tightly closed flask at -20°C until used.

Cardiolipin. Cardiolipin was obtained from Sigma (C 1649) as an ethanolic solution containing 5.0 mg cardiolipin per ml.

Sulfatide. The sulfatide used was a cerebroside sulfate from bovine brain (S 1006; Sigma).

Enzyme-linked immunosorbent assays (ELISAs). ELISAs were performed in maxisorp plates (Nunc, Roskilde, Denmark) according to standard procedures. Briefly, protein antigens (sonically extracted) were adjusted to 20 μ g per ml of saline-borate buffer (SBB, pH 8.4, containing 0.309 g sodium tetraborate per liter of 0.15 M NaCl) and distributed in 100- μ l aliquots (2 μ g protein) per well. Plates were incubated for 2 hr at 37°C and then overnight at 4°C . Mycobacterial lipids, tissue lipid antigens and bovine brain sulfatide were first dissolved in chloroform (1 mg lipid per 0.1 ml chloroform) and then diluted in absolute ethanol to 20 μ g per ml; cardiolipin was directly diluted in absolute ethanol to 20 μ g per ml. One hundred ml of the lipids were distributed per well in the ELISA plates, and the plates were left overnight at 37°C to allow evaporation of the solvent.

Then the procedure was similar for both the lipid and protein antigens and included: a) washing of the wells (twice) with SBB; b) blocking of the wells with 150 μ l of 2%

TABLE 1. *Antibodies to mycobacterial protein and lipid antigens in the serum of healthy or M. lepraemurium (MLM)-infected mice.*

MLM-antigen	Healthy mice (H) (N = 16)	MLM-mice (M) (N = 16)	p Value ^b (H vs M)
Proteins	0.034 ± 0.024 ^a	1.436 ± 0.150	<0.001 ^b
Lipids	0.014 ± 0.013	0.539 ± 0.176	<0.0001 ^b

^aMean optical density value (490 nm) ± standard error of three experiments set in triplicate. Values corrected by subtracting mean absorbencies from three wells treated with ethanol (for lipid antigens) or from wells with no antigen (for protein antigens).

^bMann-Whitney *U* test.

skim milk for 60 min at 37°C; c) the addition of the test sera (100 µl per well) diluted 1:100 in SBB, and the incubation of the plates for 60 min at 37°C. The 1:100 dilution of sera was chosen because it allowed high readings in the presence of antigen and a very low background in the absence of it; d) washing of the wells with 150 µl of SBB (three washes, 1 min each); e) the addition of 100 µl of a horseradish peroxidase-labeled rabbit anti-mouse immunoglobulin serum diluted 1:5000 in SBB and incubation for 60 min at 37°C (this dilution of the conjugated antibody allowed an optical density of 1.0_{492 nm} in the presence of 1.0 µg of mouse immunoglobulins in the ELISA procedure described); f) washing of the wells with 150 µl of SBB (four washes, 1 min each); g) the addition of 100 µl of a mixture containing 4.0 mg of *o*-phenylenediamine and 10 µl of 30% hydrogen peroxide in 10 ml of 0.01 M acetate buffer, pH

5.0; h) the incubation for 20 min at room temperature; and i) the addition of one drop of 3N sulfuric acid to arrest the reaction. Readings were made in an ELISA reader (Labsystems Multiskan Plus, Helsinki, Finland) at 492 nm. Results are given as optical density units (ODU) at the stated wavelength.

Statistic analysis. Data were first analyzed by a one-way ANOVA test or by a two-way ANOVA test, depending on the experiment. Those data that passed the test were then analyzed by either the Student's *t* test or the nonparametrical Mann-Whitney *U* test. Those data that did not pass the ANOVA tests were analyzed by either the Mann-Whitney test or the Kruskal-Wallis test, depending on the experiment. Due to the fact that an outbred line of mice (NIH) was used in this study, a stringent *p* value of less than 0.02 (*p* < 0.02) was taken as the cut-off limit of statistical significance.

RESULTS

Palsy. A small fraction of mice infected intraperitoneally with *M. lepraemurium* develop bilateral paralysis of the rear limbs. Paralysis of the front limbs has never been seen (The Figure).

Antibodies to protein antigens. Antibodies to protein mycobacterial antigens appeared at high levels in the serum of *M. lepraemurium*-infected mice (1.436 ± 0.150 ODU vs 0.034 ± 0.024 in the noninfected group, *p* = 0.001). Levels of antibodies to protein antigens served as a reliable index of infection, which was far advanced in the 16 animals included in the study (Table 1).

TABLE 2. *Antibodies to lipids of M. lepraemurium (MLM) in healthy or MLM-infected mice.*

Group	No.	LPL ^a	IPL ^b	HPL ^c
Healthy	16	0.007 ± 0.012 ^d	0.016 ± 0.002	0.014 ± 0.004
MLM-infected	16	0.014 ± 0.010	0.405 ± 0.045	0.010 ± 0.007
p Value		>0.05 ^e	<0.0001 ^f	>0.05 ^e

^aLPL = Low polarity lipids.

^bIPL = Intermediate polarity lipids.

^cHPL = High polarity lipids.

^dMean value ± standard error of absorbencies (492 nm) from three experiments set in triplicate. Values were corrected by subtracting the mean absorbencies of wells only treated with ethanol (0.104 for healthy mice, 0.048 for MLM-infected mice).

^eStudent *t* test.

^fMann-Whitney *U* test.

TABLE 3. *Isotypes of anti-M. lepraemurium antibodies in murine leprosy.*

Antigen	Antibody class		p Value
	IgM	IgG	
Proteins	0.300 ± 0.191 ^a	0.500 ± 0.213	0.05 ^b
Lipids	0.600 ± 0.136	0.262 ± 0.100	<0.001 ^c

^aMean values ± standard error of assays set in triplicate. Values were corrected by subtracting the mean absorbencies from three wells only treated with ethanol.

^bStudent *t* test.

^cMann-Whitney *U* test.

Antibodies to *M. lepraemurium*-derived lipids. As shown in Table 1, mice infected with *M. lepraemurium* also contained very significant levels of antibodies to the mycobacterial lipids (0.539 ± 0.176 ODU vs 0.014 ± 0.013 in the healthy group); antibodies, however, were almost entirely directed to the lipids of intermediate polarity, mostly glycolipids (0.405 ± 0.045 ODU vs 0.014 ± 0.010 for low polarity lipids and 0.010 ± 0.007 for highly polar lipids) (Table 2). This finding corroborates our previously reported results in regard to the high immunogenicity of this lipid family (¹⁴).

Isotypes of antibodies to *M. lepraemurium*-derived antigens. Although *M. lepraemurium*-infected animals contained large amounts of antibodies to both lipid and protein mycobacterial antigens, most antibodies to lipid antigens belonged to the IgM class (0.600 ± 0.136 ODU vs 0.262 ± 0.100 for IgG, *p* < 0.001), while the majority of antibodies to protein antigens were IgG (0.500 ± 0.213 vs 0.300 ± 0.192 for IgM, *p* = 0.05) (Table 3).

Antibodies to phospholipids and other non-mycobacterial lipids. *M. lepraemurium*-infected mice produce significant amounts of antibodies to mycobacterial lipids but do not harbor antibodies to lipids extracted from normal spleen and liver, normal brain, cardiolipin or sulfatide. Normal animals do not contain significant amounts of antibodies to any of the lipids used, including those lipids of *M. lepraemurium* (Table 4).

DISCUSSION

As protein antigens, *M. lepraemurium*-derived lipids are immunogenic in the mouse. However, based solely on the absorbency values found in the ELISAs, it seems that proteins are more immunogenic than the lipid antigens of the microorganism (a basic principle of the immunology). Also, as expected, protein antigens led to the preferential production of IgG antibodies, while lipid antigens preferentially stimulated production of IgM antibodies (another basic principle of immunology). Lipid antigens might function as T-cell independent antigens able to directly stimulate B lymphocytes; T-cell-independent humoral responses are basically made up of IgM antibodies since the change of class (switch) usually does not occur. Recently, a novel set of non-MHC-related molecules (CD1 molecules) expressed prominently by human antigen-presenting cells (macrophages, dendritic cells and a subset of B cells) have been implicated in the presentation of "unusual" mycobacterial antigens to CD1-restricted T cells (²). Some CD1 molecules have been shown to restrict antigen-specific

TABLE 4. *Antibodies to several lipids in murine leprosy.*

Lipid source	Mouse group (16 each)		p Value (H vs M)
	Healthy (H)	MLM-infected (M)	
Purified MLM	0.012 ± 0.009 ^a	0.450 ± 0.171	<0.001 ^b
Mouse liver and spleen	0.043 ± 0.004	0.047 ± 0.005	>0.6 ^b
Mouse brain	0.044 ± 0.034	0.038 ± 0.019	>0.9 ^b
Cardiolipin	0.110 ± 0.045	0.119 ± 0.041	>0.5 ^c
Sulfatide	0.064 ± 0.094	0.034 ± 0.033	>0.5 ^b

^aMean absorbency ± standard error (ODU at 492 nm) of assays set in triplicate. Values were corrected by subtracting the mean absorbency of wells treated with only ethanol.

^bMann-Whitney *U* test.

^cStudent *t* test.

T-cell responses, being CD1c-restricted T-cell lines, able to recognize mycobacterial lipid antigens (1). In the mouse, molecules analogous to CD1 have been described (3) but their role has not been (yet) fully explored. A similar murine CD1c-molecule might participate in the presentation of mycobacterial lipid antigens to CD1-restricted T cells, but the consequence of this interaction in regard to the class of immunoglobulin (IgG or IgM) favored is not yet known.

Regardless of the high levels of antibodies to mycobacterial lipids in *M. lepraemurium*-infected mice, these animals did not contain antibodies reactive to non-mycobacterial lipids such as those isolated from the liver, spleen and brain of normal mice, nor antibodies to cardiolipin. At first sight, this would seem a contradictory result in light of the reports on human leprosy that indicate a high frequency of antibodies to lipids of the nervous system (4, 5) and to cardiolipin (7-10), both lipid preparations rich in phospholipids. Our findings, however, are in agreement with the practically null clinical involvement of nerve damage in murine leprosy. Even those animals that showed bilateral paralysis of the rear limbs (2 out of the 16 tested) lacked antibodies to non-mycobacterial lipids.

Although anticardiolipin and antineural antibodies are frequently present in multibacillary (MB) and less frequently in paucibacillary (PB) leprosy, their rise not always correlates with cardiovascular involvement, bacterial index, active neuritis, or nerve enlargement (5, 17). Others, however, have found that MB patients also have elevated levels of IgM antibodies to cerebroside sulfatide, and that these antibodies fell at the onset of erythema nodosum leprosum, suggesting a participation of these antibodies in the pathology of reactional leprosy (18). Our data, however, do not reveal the presence of significant levels of antibodies to cerebroside sulfatide in the sera of *M. lepraemurium*-infected animals (nor in the sera of normal animals).

Thus, autoimmunity to lipid antigens in murine leprosy does not seem to be a feature of the disease, this correlating with the lack of nerve involvement in this illness. Bilateral paralysis observed in some animals (1% to 3%) infected with *M. lepraemurium*

might indicate active parasitization of nerves rather than a consequence of autoimmunity. This possibility, however, deserves further investigation.

SUMMARY

In order to know whether antibodies to phospholipids and other host lipids play a role in the pathology of murine leprosy, we looked for the presence of antibodies to cardiolipin, cerebroside sulfatide, and to lipids extracted from normal murine spleen, liver and brain in the sera of mice bearing a 6-month infection with *Mycobacterium lepraemurium*. We also looked for the presence of antibodies to lipids isolated from *M. lepraemurium*. We found that all of the 16 animals examined contained high levels of antibodies to the mycobacterial lipids of intermediate polarity (mostly glycolipids) but none of them had antibodies to the other lipids tested, including those isolated from mouse liver, spleen and brain, bovine cardiolipin and sulfatide, nor any significant levels of antibodies to mycobacterial lipids of high or low polarity. The infected animals also had high levels of antibodies to antigens sonically extracted from the microorganism. Antibodies to the socially extracted antigens (mostly proteins) were mainly IgG, while antibodies to the lipid antigens were predominantly IgM. Despite the low but significant percentage (1%–3%) of infected animals developing bilateral paralysis of the rear limbs, autoimmunity (due to antibodies to phospholipids and other host lipids) does not seem to be a feature of murine leprosy.

RESUMEN

Para saber si los anticuerpos contra fosfolípidos y otros lípidos del huésped juegan algún papel en la patología de la lepra murina, investigamos la presencia de anticuerpos contra cardiolipina y cerebroside sulfatido, y contra lípidos de hígado, bazo y cerebro de ratón normal, en el suero de ratones con 6 meses de infección por *Mycobacterium lepraemurium* (MLM). También buscamos la presencia de anticuerpos contra lípidos aislados de MLM. Los 16 animales estudiados contuvieron niveles elevados de anticuerpos contra los lípidos de MLM de polaridad intermedia (glicolípidos, en su mayoría), pero ninguno de ellos tuvo niveles significativos de anticuerpos contra los otros lípidos usados en el estudio, incluyendo los lípidos de hígado, bazo y cerebro de ratón, cardiolipina y sulfátido. Los

animales infectados tampoco tuvieron niveles significativos de anticuerpos contra los lípidos micobacterianos de alta y de baja polaridad, pero sí tuvieron cantidades elevadas de anticuerpos contra los antígenos liberados por sonicación del microorganismo (principalmente proteínas). Los anticuerpos contra los antígenos proteicos fueron mayoritariamente de la clase IgG mientras que los anticuerpos contra los antígenos lipídicos fueron predominantemente IgM. No obstante que un porcentaje bajo pero significativo (1–3%) de los animales infectados desarrollan parálisis bilateral de las extremidades traseras, la autoinmunidad (por anticuerpos contra fosfolípidos y otros lípidos del huésped) no parece ser una característica de la lepra murina.

RÉSUMÉ

En vue de savoir si les anticorps contre les phospholipides et autres lipides de l'hôte jouent un rôle dans la pathologie de la lèpre murine, nous avons examiné, dans le sérum de souris infectées depuis 6 mois par *Mycobacterium lepraemurium*, la présence d'anticorps contre la cardiolipine, les cérebrosides sulfatés et les lipides extraits à partir de la rate, du foie et du cerveau de souris normales. Nous avons également recherché la présence d'anticorps contre des lipides isolés à partir de *Mycobacterium lepraemurium*. Nous avons trouvé, chez les 16 souris examinées, de fort taux d'anticorps contre les lipides mycobactériens de polarité intermédiaire (principalement des glycolipides). Cependant, aucun de ces animaux n'avait d'anticorps contre les autres lipides testés tels ceux isolés de la rate, du foie et du cerveau de souris normale, la cardiolipine bovine et cérebrosides sulfatés, ainsi que une absence de niveaux significatifs d'anticorps contre les lipides de polarité haute ou basse. Les animaux infectés avaient aussi de hauts niveaux d'anticorps contre les antigènes extraits des micro-organismes par ultrason aux ultrasons. Les anticorps dirigés contre les antigènes extraits par ultrason (principalement des protéines) étaient principalement des IgG, tandis que les anticorps dirigés contre les antigènes lipidiques étaient principalement des IgM. En dépit du pourcentage bas mais significatif (1%–3%) d'animaux infectés présentant des paralysies bilatérales des pattes arrières, l'auto-immunité (due à des anticorps dirigés contre les phospholipides et autres lipides de l'hôte) ne semble pas être une caractéristique de la lèpre murine.

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