Lepr Rev (1986) **57,** 317–327

Antimycobacterial antibodies in *Dasypus novemcinctus* infected with *Mycobacterium leprae* and their correlation with the serum levels of lactate dehydrogenase

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Accepted for publication 11 June 1986

Summary Discovery of armadillos (*Dasypus novemcinctus*) as animals susceptible to infection with *Mycobacterium leprae*, has allowed the experimental study of leprosy to extend beyond the limits of the mouse footpad. Armadillos, however, do not all become equally infected with a given dose of *M. leprae*. Therefore, it would be advantageous to establish a technique for the early identification of those animals bearing the disease.

Infection in armadillos originates systemic involvement which includes liver damage and the consequent release of LDH into circulation before the appearance of the clinical signs of the disease. In this study, where an enzyme-linked immunoassay for the detection of antimycobacterial antibodies was developed, those very same animals that showed an increase in their serum LDH activity showed the presence of anti-*M. leprae* antibodies to significant titres and eventually the presence of disease. From the results with some animals, it appears that the presence of antimycobacterial antibodies occurs before the elevation in the serum LDH activity.

Periodical measurement of both antimycobacterial antibodies and LDH activity in the sera of *M. leprae*-inoculated armadillos may help one to detect the early infection, decide whether or not an animal is indeed infected, and decide how to proceed with the animals under investigation. The results also reveal some of the immunological and biochemical consequences of the *M. leprae*-infection in the armadillo.

Introduction

It has been previously demonstrated[17,3] that mice infected with *Mycobacterium lepraemurium* develop alterations in their serum levels of lactate dehydrogenase

0305-7518/86/057317+11 $01.00

(LDH), glutamate-oxalacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT). Such alterations, visualized as increments in the above enzyme activities, were proportional to the time and extent of the infection up to a certain limit, and appeared earlier than the clinical signs of the disease. This finding was conceived as a means to monitor the progress of the mycobacterial disease. In a subsequent study,[18] similar enzyme alterations in the serum of armadillos (*Dasypus novemcinctus*) inoculated at variable times with *M. leprae* were looked for and here again we observed a good correlation between the extent of the disease and the increments in the serum levels of LDH, GOT and GPT activities. Although the increments in the levels of GOT and GPT appeared earlier than the elevations in the level of LDH, the latter activity showed the most striking increments. Increases in the serum LDH levels were also observed under conditions where significant tissue damage was present, as in the case of severe and extended pyogenic infections. However, when the increase in the serum LDH activity was related to leprosy, the isozyme LDH-V was the one involved, suggesting liver damage. The liver, both in mice and armadillo, is a very susceptible organ to the attack by the corresponding mycobacteria.

In this communication, we provide evidence that, under controlled conditions to avoid unwanted infections, increments in serum LDH activity are indeed related to the leprosy infection in armadillos. This was established by comparing the serum LDH increments with the clinical and bacteriological evidences of the disease and with the presence in serum of antibodies against *M. leprae*. Antimycobacterial antibodies were detected by means of an enzyme-immunoassay developed in our laboratory.

Materials and methods

ARMADILLOS (*DASYPUS NOVEMCINCTUS*) AND THEIR SERA

The sera from 12 surviving (out of 15) armadillos which had been previously inoculated by the intravenous route with 10^8 *M. leprae* and the sera from 12 other noninoculated animals were examined for the presence of antimycobacterial antibodies and LDH activity. Details on the armadillos (origin, housing, inoculation, follow up, etc.) and on the source and isolation of bacilli, have been given elsewhere.[18] The data on the evidences of infection came from the files of Dr F. Quesada-Pascual (Department of Immunology, ENCB, IPN), who was conducting work simultaneously on other aspects of infected armadillos. Such evidences of infection were assessed by periodical physical examination and by the search of acid-fast bacilli (AFB) in smears made from nasal exudate, cutaneous lymph and ears' imprints.

PURIFICATION OF RABBIT GAMMAGLOBULIN ANTI-ARMADILLO GAMMAGLOBULIN

Rabbit anti-armadillo gammaglobulin antibody (anti-AGG Ab) was prepared by passing hyperimmune serum (produced in our laboratory) through an Affigel-10 column (Bio-rad) to which armadillo gammaglobulin had been covalently linked following the manufacturers' directions and according to standard procedures.[5] Quantitation of proteins in this and other solutions was by the Lowry's method.[13]

PEROXIDASE LABELLING OF ANTI-AGG AB

This was carried out by using the two-step glutaraldehyde method of Avrameas and Ternynck.[1] The labelled-antibody preparation (1 mg of antibody per ml of PBS) was stabilized with 100 mg per ml of bovine serum albumin, sterilized through 0·45 μm millipore, and frozen or lyophilised in aliquots until used.

ANTIGENS

Mycobacterium leprae was purified by Draper's method[2] from the liver of a heavily infected armadillo injected 12 months before with 10^8 *M. leprae* from an untreated case of human leprosy. *M. lepraemurium* was isolated by Prabhakaran's method[15] from the subcutaneous lepromata of NIH mice bearing a 6-month infection with *M. lepraemurium*, Hawaiian strain.

M. leprae and *M. lepraemurium* soluble antigens were prepared by sonic disruption of bacillary suspensions containing about 1×10^9 bacteria per ml by using a cell disruptor (Heat systems, ultrasonic, model W370, N.Y.) at 140 watts, for an effective time of 30 min. Sonicates were dialysed against PBS, centrifuged to eliminate insoluble debris, and then sterilized through 0·45 μm millipore. Working solutions were made in plain distilled water to contain 40 μg protein per ml.

ENZYME-LINKED IMMUNOASSAY (ELISA)

This was performed under standard conditions by using 2 μg of *M. leprae*- or *M. lepraemurium*-derived protein in 50 μl of water per well of Immunolon 2 Removawell strips (Dynatech), 0·1 ml of PBST (phosphate buffered saline, pH 7·1, with 0·05% tween 20) for the washings, 50 μl of armadillo sera diluted 1 : 100 or 1 : 1000 in PBS, 50 μl of PO-labelled antibody diluted 1 : 500, 50 μl of a mixture containing 4·0 mg of o-phenylene diamine (Sigma) and 10 μl of 30% H_2O_2 in 10 ml of 0·025 M citrate-0·05 M phosphate buffer, pH 5·4, 20 μl of 8 N H_2SO_4 to stop the reaction, and reading at 490 nm in a minireader II densitometer (Dynatech).

LACTIC DEHYDROGENASE ACTIVITY AND ISOZYMES

Lactic dehydrogenase (LDH) in serum was assayed according to the principles of Wroblewski and LaDue[20] by using commercial kits (LDH-UV-System, Boehringer Mannheim, GMBH, Germany), and LDH isozymes were quantitated by electrophoresis on cellulose acetate following the technique of Mager *et al.*[14]

Results

ANTIMYCOBACTERIAL ANTIBODIES USING *M. LEPRAE* AS THE ANTIGEN

Figure 1 depicts the results when 2 dilutions of armadillo sera (1:100 and 1:1000) and soluble *M. leprae* antigens were used in the assay. Sera from 12 healthy uninfected animals gave a mean OD reading ($\bar{X}$) of 0·24 with an SD of $\pm$0·04 at the 1:100 dilution, and 0·12 $\pm$ 0·04 at the 1:1000 dilution. It can be seen that five sera from *M. leprae*-inoculated armadillos (A03, A04, A08, A09 and A11) gave a

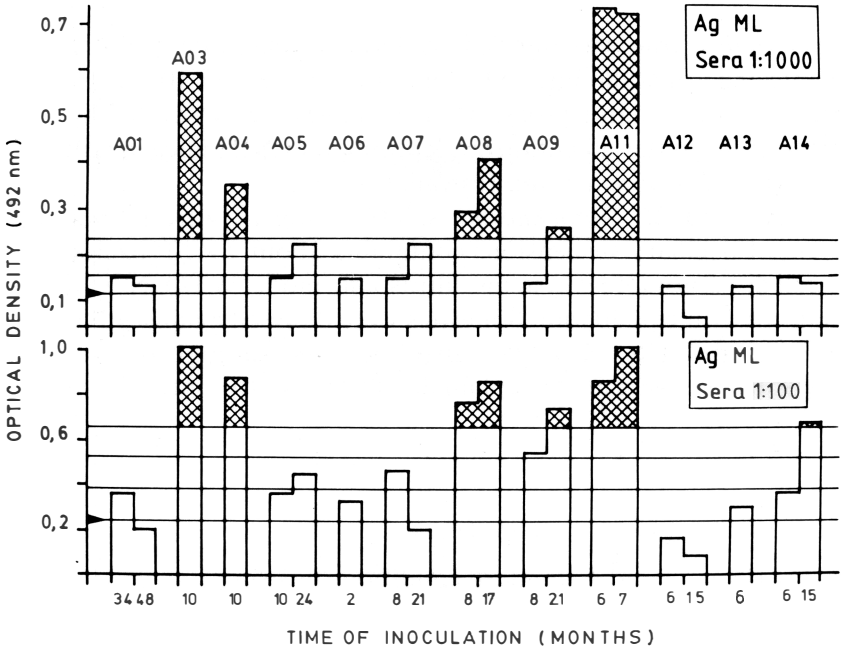

Figure 1. Antimycobacterial antibody activity in the sera of 12 armadillos previously inoculated with 10^8 *M. leprae* by the intravenous route. Sera from control and inoculated animals were tested at a 1:1000 (upper panel) or 1:100 (lower panel) dilution. The antigen was a sonicate prepared from *M. leprae* (2 μg protein per well). Arrow heads at the left indicate the mean normal value found in a group of 12 noninoculated animals; horizontal lines above this value correspond to one, two, and three SD over the mean.

Table 1. Presence of antimycobacterial antibodies, clinical signs of disease and acid-fast bacilli in nasal exudate and/or ear's imprints, in armadillos inoculated with *Mycobacterium leprae*

Animal	Months of inoculation	Clinical† signs of disease	AFB‡	Serum antibodies reactive to soluble antigens from§ *M. leprae* 1:100 $\bar{X}+2$ SD	*M. leprae* 1:100 $\bar{X}+3$ SD	*M. leprae* 1:1000 $\bar{X}+2$ SD	*M. leprae* 1:1000 $\bar{X}+3$ SD	*M. lepraemurium* 1:100 $\bar{X}+2$ SD	*M. lepraemurium* 1:100 $\bar{X}+3$ SD	*M. lepraemurium* 1:1000 $\bar{X}+2$ SD	*M. lepraemurium* 1:1000 $\bar{X}+3$ SD
A 01	34 (H)*	No	No	−	−	−	−	−	−	−	−
A 01	48	No	No	−	−	−	−	−	−	−	−
A 03	10 (H)	Yes	Yes	+	+	+	+	+	+	+	+
A 04	10 (H)	Yes	Yes	+	+	+	+	−	−	+	+
A 05	10 (H)	No	No	−	−	−	−	−	−	−	−
A 05	24	No	No	−	−	+	−	−	−	−	−
A 06	2 (A)	No	No	−	−	−	−	−	−	−	−
A 07	8 (H)	No	No	−	−	−	−	−	−	−	−
A 07	21	No	No	−	−	+	−	−	−	−	−
A 08	8 (H)	No	No	+	+	+	+	+	−	+	−
A 08	17	Yes	Yes	+	+	+	+	+	+	+	+
A 09	8 (H)	No	No	+	−	−	−	−	−	−	−
A 09	21	No	Yes	+	+	+	+	+	+	+	+
A 11	6 (A)	No	No	+	+	+	+	+	−	+	+
A 11	7	Yes	Yes	+	+	+	+	+	−	+	+
A 12	6 (A)	No	Yes**	−	−	−	−	−	−	−	−
A 12	15	No	No	−	−	−	−	−	−	−	−
A 13	6 (A)	No	No	−	−	−	−	−	−	−	−
A 14	6 (A)	No	No	−	−	−	−	−	−	−	−
A 14	15	No	Yes	+	+	−	−	+	−	+	−

* Each armadillo was inoculated, intravenously, with about 10^8 *M. leprae* from human (H) or armadillo (A) origin.

† Clinical signs of disease include the appearance in the skin of nodules or erythematous lesions, swelling of lymph nodes, emaciation, and some times epistaxis.

‡ Acid-fast bacilli were looked for in Ziehl–Neelsen stained smears made from nasal exudate and from the lymph exuded from skin scrapings and ears' cuts.

§ Positive (+) results are those OD readings higher than the mean value plus two or three standard deviations ($\bar{x}+2$ SD or $\bar{x}+3$ SD) found in a control, noninoculated group. These mean and SD values at each dilution of sera tested were $0{\cdot}24 \pm 0{\cdot}14$ (1:100) and $0{\cdot}12 \pm 0{\cdot}04$ (1:1000) with *M. leprae* soluble antigens, and $0{\cdot}47 \pm 0{\cdot}19$ (1:100) and $0{\cdot}18 \pm 0{\cdot}07$ (1:1000) with *M. lepraemurium* soluble antigens.

** One single AFB was found in the nasal exudate of armadillo A 12.

positive result (OD readings higher than $\bar{X}+3$ SD) at the two dilutions tested. This is in good agreement with the other parameters of infection (Table 1).

ANTIMYCOBACTERIAL ANTIBODIES USING *M. LEPRAEMURIUM* AS THE ANTIGEN

Similar results to the ones described above were obtained when *M. lepraemurium* sonicate was used as the antigen. However, here the mean normal and SD values for the dilutions of the sera tested were $0{\cdot}47 \pm 0{\cdot}19$ (dilution 1:100) and $0{\cdot}18 \pm 0{\cdot}07$ (dilution 1:1000). As above, taking as the limit the corresponding mean normal value plus 3 SD, only sera A03, A04, A08, A09 an A11 remained positives at the 1:1000 dilution which, once again, resulted more discriminative than the 1:100 dilution.

CORRELATION BETWEEN CLINICAL AND/OR BACTERIOLOGICAL SIGNS OF DISEASE, AND THE PRESENCE OF ANTIMYCOBACTERIAL ANTIBODIES

None of the eight armadillos for which data at two different postinoculation times are shown (Table 1) showed clinical or bacteriological evidences of disease at the time of the first examination reported. However, such evidences of disease were apparent in two animals (A08 and A11) when the latest examination was performed. These two animals were serologically positives from the first examination despite the lack of clinical or bacteriological signs of disease at this time.

As for the animals for which data at a single postinoculation time are given, there were two of them (A03 and A04) that showed clinical, bacteriological and serological evidences of infection, and two others (A06 and A13) that were negatives by all criteria. Armadillos A09 and A14 were clinically and bacteriologically negatives at the time of the first examination and, accordingly, they were serologically negatives at this time. At the latest date of sampling, A09 and A14 were bacteriologically and serologically positives but remained free of clinical signs of disease. A09 gave more consistent positive results than A14 in which positive results were limited to the lower dilution of the serum (1:100). Probably A14 was an animal bearing an early disease.

SERUM DILUTIONS 1:100 *VS* 1:1000.

Within each antigen system (*M. leprae* or *M. lepraemurium*), the results with the 1:1000 dilution of the test sera were somewhat more discriminative than those results with the 1:100 dilution (Table 1). Perhaps this is due to the lower background values found in the control, noninoculated, group of armadillos at the higher dilution of the sera (see above). Within the infected animals, some of

them gave OD readings as high as 0·80 at the 1 : 1000 dilution with either antigen preparation.

When the mean normal value plus 3 SD of the mean (instead of the mean value plus 2 SD) was taken as the upper limit of negativity, the number of positive results diminished leaving without change only those sera that were absolutely positives (Table 1).

MYCOBACTERIUM LEPRAE VS M. LEPRAEMURIUM ANTIGENS

From Figure 2, it is clear that both mycobacterial antigen preparations were reactive with the sera of exactly the same animals. However, levels of antibody against the *M. leprae* sonicate correlated better with the other parameters of infection.

ANTIMYCOBACTERIAL ANTIBODIES *VS* LDH LEVELS

Due to technical reasons, simultaneous determinations of antimycobacterial antibodies and LDH activity were performed in serum samples that had been

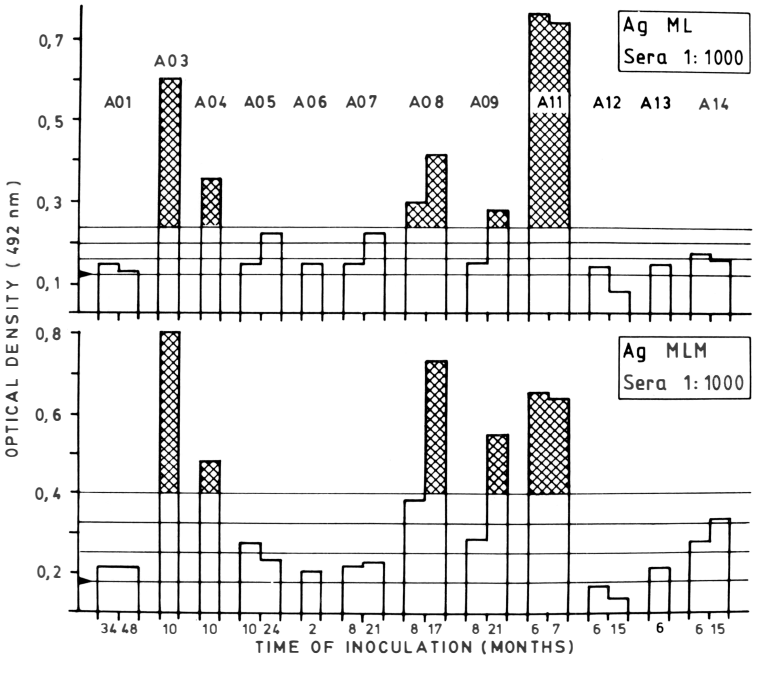

Figure 2. Anti-*M. leprae* antibody activity in the sera of 12 armadillos previously inoculated with 10^8 bacilli by the intravenous route. Control and test sera were assayed diluted 1 : 1000 against 2 μg of protein from either *M. leprae* (upper panel) or *M. lepraemurium* (lower panel) sonicates. Arrow heads at the left indicate the mean normal value found in a group of 12 noninoculated animals and the horizontal lines above this value correspond to one, two, and three SD over the mean.

kept frozen at −70°C for several months. As a significant loss of LDH activity was observed in those sera, it was decided to look at their LDH-isozymes patterns. Comparing with zymograms from normal fresh sera, it was observed that LDH-isozymes IV and V were the most unstable, completely disappearing from the frozen sera, that isozymes I and II were variably affected, and that LDH-isozyme III was practically unaffected by the long lasting storage at −70°C. Fortunately (and opposed to what we had previously thought),[18] LDH isozyme III was proportionally altered by the leprosy infection, this allowing us to compare LDH activity with the presence of antimycobacterial antibodies at variable postinoculation times.

As it is observed from Figure 3, there was an almost absolute correlation between abnormally increased levels of serum LDH activity and meaningful titres of antimycobacterial antibodies. This figure illustrates only those results from

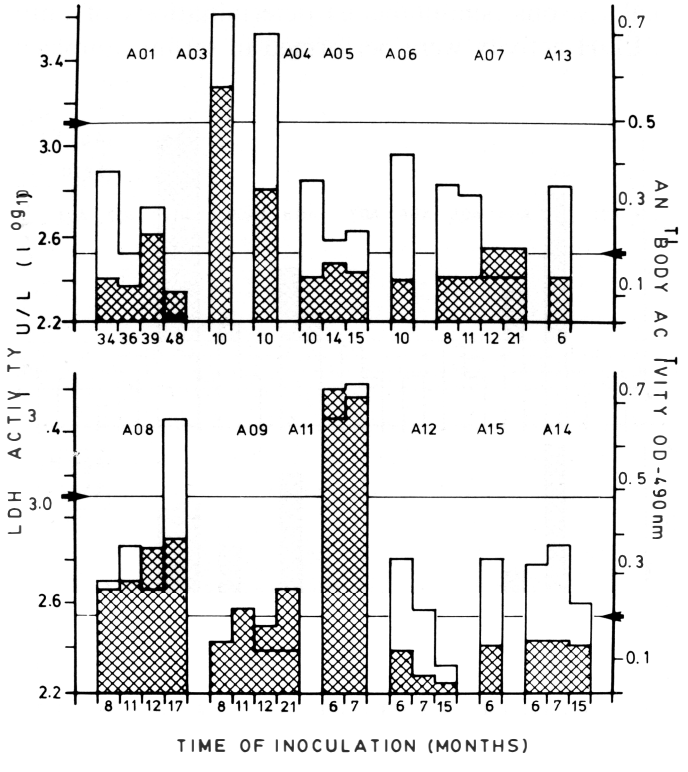

Figure 3. Anti-*M. leprae* antibody activity (hatched bars) and LDH isozyme-III activity (open bars) in the sera of 13 armadillos at different postinoculation times. Arrows at the left indicate the mean plus 2 SD value (1258 U/l) for LDH isozyme-III activity in a normal group of 12 animals; arrows at the right indicate the mean plus 2 SD value (OD = 0·20) for the antimycobacterial antibody activity in the same group of normal animals.

serum samples on which both determinations were simultaneously performed. There were several cases (A01, A05, A06, A07, A09, A12, A13, A14 and A15) where both parameters were within the normal limits at the different times studied (the mean normal plus 2 SD value for LDH isozyme III was 1258 U/l). There was, however, one case (A08) where antimycobacterial antibodies were above those normal limits, nine months before the increase in the LDH activity. This suggested that the development of antimycobacterial antibodies might precede the abnormal release of LDH into circulation. Serum samples from armadillos A03, A04, A08 (at 17 months postinoculation) and A11, showed both increased titres of antimycobacterial antibodies and abnormally elevated levels of LDH. These were the very same animals that had developed (A03 and A04) or that later on developed (A08 and A11) the clinical and bacteriological signs of the disease.

Discussion

Since 1971, nine-banded armadillos have been the most suitable animals for the experimental study of leprosy.[11] Several studies have been done on different aspects of this animal model including histopathology, susceptibility, immunology, biochemistry, and some others.[7–10] There are reports on the liver involvement in armadillos with both experimental and natural leprosy[6,12,19] and also, it has been reported that proportional to the liver damage there is a release into circulation of several enzyme activities (LDH, GOT and GPT).[18] This enzyme release results from the progressive parenchymal infiltration by lepromatous granulomas that leads to the damage of the hepatocytes. From the above-mentioned enzyme activities, the one belonging to LDH showed the most striking increments (from 4099 ± 269 U/l in noninoculated animals to 18500 U/l in leprosy-infected animals). We thought of this finding as a useful tool to monitor the progress of the infection. This biochemical marker of infection was, however, nonspecific, as LDH activity also increased as a result of other nonleprosy related infections. When care was taken to avoid unwanted infections, increments in LDH activity were related to leprosy and were measurable well before the appearance of the clinical signs of the disease.[18] In a similar study on the biochemical alterations induced by the experimental infection of armadillos with *M. leprae*,[16] it was found that mean angiotensin-converting enzyme values were significantly elevated in animals with lepromatoid leprosy. As the degree of elevation roughly paralleled the extent of the infection, the authors felt that the serum angiotensin-converting enzyme assay could be of value for evaluating armadillos for spontaneous or experimentally induced infection with *M. leprae* (the degree of elevation was considered as an approximate index of the extent of infection). In the same study, the authors were unable to see any difference between healthy and infected armadillos in regard to two other serum enzymes:

transcobalamin and lysozyme (this latter enzyme was found only in negligible amounts in either healthy or lepromatoid animals).

On the other hand, as in human beings, armadillos infected with *M. leprae* develop high amounts of antibodies against the microorganism.[4] Antimycobacterial antibodies appeared at measurable titres in those animals in contact with mycobacteria and their titres increased proportionally to the bacterial load in the animals that developed leprosy. By using a sensitive enzyme-immunoassay (ELISA) we could demonstrate the presence of antimycobacterial antibodies at titres that were well above the mean value (plus 3 SD) found in the serum of noninoculated animals; these same sera contained elevated levels of LDH activity. Both *M. leprae* and *M. lepraemurium* sonicates were appropriate antigen preparations for the detection of antimycobacterial antibodies, though results with *M. leprae* sonicate correlated better with the other parameters of infection. In the only other paper we know on the detection of anti-*Myobacterium leprae* (antigen 7) antibodies in armadillos,[4] a radioimmunoassay was developed and applied aiming to detect a preexisting natural infection and to identify those animals developing a progressive disease. Despite the different systems used (RIA *vs* ELISA; antigen 7 *vs* a whole sonicate), the results were concordant: in both studies, the dilution 1:1000 of the sera gave the greatest differences between individual samples: they found that 14 out of 17 animals with systemic infection after inoculation with *M. leprae*, showed increased antibody activity; we found that the five animals (out of 13 inoculated with *M. leprae*) that showed an increased antibody activity were the same animals that eventually developed a systemic disease.

As we found an almost absolute correlation between titres of antimycobacterial antibodies, levels of LDH activity, and presence and extent of the disease, these findings, apart from their potential application in the monitoring of the experimental disease under a variety of circumstances, may contribute to the better understanding of the immunobiochemical changes induced by the leprosy bacillus in one of the most adequate animal models for the study of leprosy, the nine-banded armadillo *Dasypus novemcinctus*.

Acknowledgments

Authors are indebted to Dr S Estrada-Parra, head of our Department of Immunology, for his continuous interest in our work and for supporting it with funds derived from several sources. Particular support came from CONACYT, Mexico, and from the DGI, IPN, to the project: Fagocitosis en lepra humana y murina. We are also indebted to Dr Jose E Garcia who prepared one of the two PO-Antibody conjugates used in this study.

O Rojas-Espinosa is a fellow from COFAA, IPN, and from the SNI, Mexico.

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