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Armadillo IgG and IgM Antibody Responses to Phenolic Glycolipid-I During Experimental Infection with *M. leprae*[1,2]

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The armadillo, when experimentally inoculated with *Mycobacterium leprae,* develops a disseminated infection similar in many respects to human lepromatous leprosy ([6, 7]). In serological studies of leprosy, armadillos have advantages over human subjects in that a number of armadillos can be inoculated at the same time with known quantitites of *M. leprae* and their serological responses can be studied over time in the absence of drug therapy. However, studies related to the immunological responses of *M. leprae*-infected armadillos have been few and, to date, we do not have a concise understanding of the humoral antibody responses of armadillos to *M. leprae.* Using a radioimmunoassay, Harboe, *et al.* ([3]) demonstrated increased amounts of antibodies to *M. leprae* antigen 7 during the course of experimental infection. In immunoblotting studies using ^{125}I-labeled protein A, Chackrabarty, *et al.* ([1]) have shown the presence of antibody to several antigenic components of *M. leprae* in the sera of infected armadillos. Recently, Truman, *et al.* ([10]) have shown that estimations of armadillo IgM antibodies to an *M. leprae*-specific antigen, phenolic glycolipid-I (PGL-I) ([4]), can be useful in following the course of the infection.

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

MATERIALS AND METHODS

Animals. Nine-banded armadillos (*Dasypus novemcinctus*) were captured within a 30-mile radius of the Gillis W. Long Hansen's Disease Center (GWLHDC), Carville, Louisiana, U.S.A. The animals were screened for wild-type infection with mycobacteria once a month for 3 months by examining the buffy coats of peripheral blood and ear snips for the presence of acid-fast bacilli (AFB). Animals found to be negative for AFB were then inoculated with *M. leprae.* Eleven armadillos were inoculated intravenously with 5×10^8 armadillo-passaged *M. leprae.* Plasma samples were collected prior to inoculation (day 0) and at approximately 3-month intervals for a period of 1 year. The samples were stored at −20°C until tested.

The 11 animals were inoculated for the purpose of *M. leprae* production. The 3-month sampling frequency is routine and is based upon the likelihood that more fre-

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quent sampling might kill the animals prematurely. These animals were sacrificed when they exhibited a heavy dissemination of *M. leprae* infection based on histological examination of ear biopsies. Plasma samples were analyzed up to approximately 1 year after inoculation.

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

Attempts at directly conjugating peroxidase molecules to rabbit anti-armadillo γ-chain proved unsatisfactory. Therefore, for detection of armadillo IgG antibodies to PGL-I, 50 μl of rabbit anti-armadillo IgG (γ-chain specific) diluted 1:1000 in 1% BSA-PBS was added subsequent to the primary antibody and washing steps. The plates were incubated at RT for 1 hr. After washing, 50 μl of peroxidase-conjugated goat anti-rabbit IgG (Cappel Laboratories, Downington, Pennnsylvania, U.S.A.) at 1:1000 dilution was added per well for 1 hr. After washing the plates, 50 μl at 0.04 mg/ml solution of ortho-phenylenediamine (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) containing 0.02% H_2O_2 in 0.02 M sodium acetate buffer, pH 5.5, was added to each well. The plates were incubated at RT for 10 min, and the reaction was stopped by adding 5 N HCl (50 μl/well). Absorbance at 492 nm was read with a spectrophotometer (Titertek Multiscan; Flow Laboratories, Richmond, Virginia, U.S.A.).

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

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

RESULTS

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

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

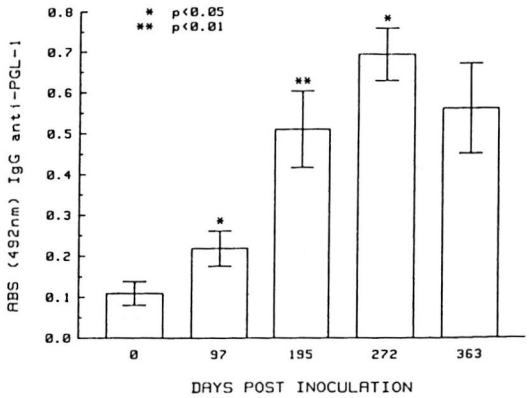

FIG. 1. IgG anti-PGL-I mean ± S.E.M. absorbance values of 11 armadillos during course of experimental infection. Asterisks indicate values significantly different from preceding values (paired *t*-test).

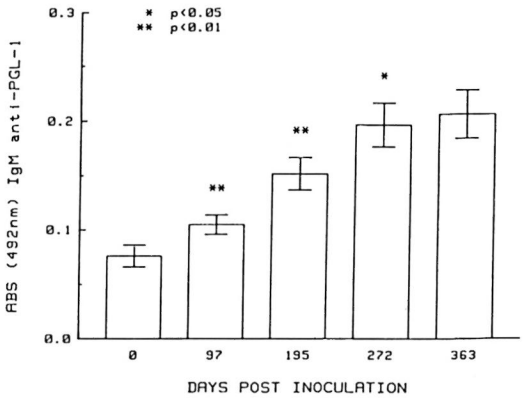

FIG. 2. IgM anti-PGL-I (see Fig. 1 legend).

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

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

DISCUSSION

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

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

Previous studies have shown that armadillos less susceptible to infection with *M. leprae* exhibit a positive lepromin skin test ([5]). These animals also showed a positive skin test in response to PGL-I, suggesting that PGL-I preparations are capable of eliciting a T-cell response in the armadillo. In human studies, lymphocyte proliferation to PGL-I was shown to be positively correlated with IgG antibodies to PGL-I ([8]), also implying that T cells are involved in the response to PGL-I. Furthermore, Levis, *et al.* ([9]) have reported high IgG anti-PGL-I in some patients with tuberculoid (BT) leprosy, a form of leprosy associated with relative control of the infection.

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

THE TABLE. *Comparison of IgG absorbance values to PGL-I with longevity and time until the appearance of AFB in ear biopsies among 11 armadillos inoculated with* M. leprae.

Armadillo group	IgG ELISA at 365 days post-inoculation	Longevity (days)	Time of appearance of AFB in ear biopsy (days)
		(Mean ± S.E.M.)	
A (N = 5)	>0.7	735 ± 162	399 ± 61
B (N = 6)	<0.7	422 ± 37	288 ± 26

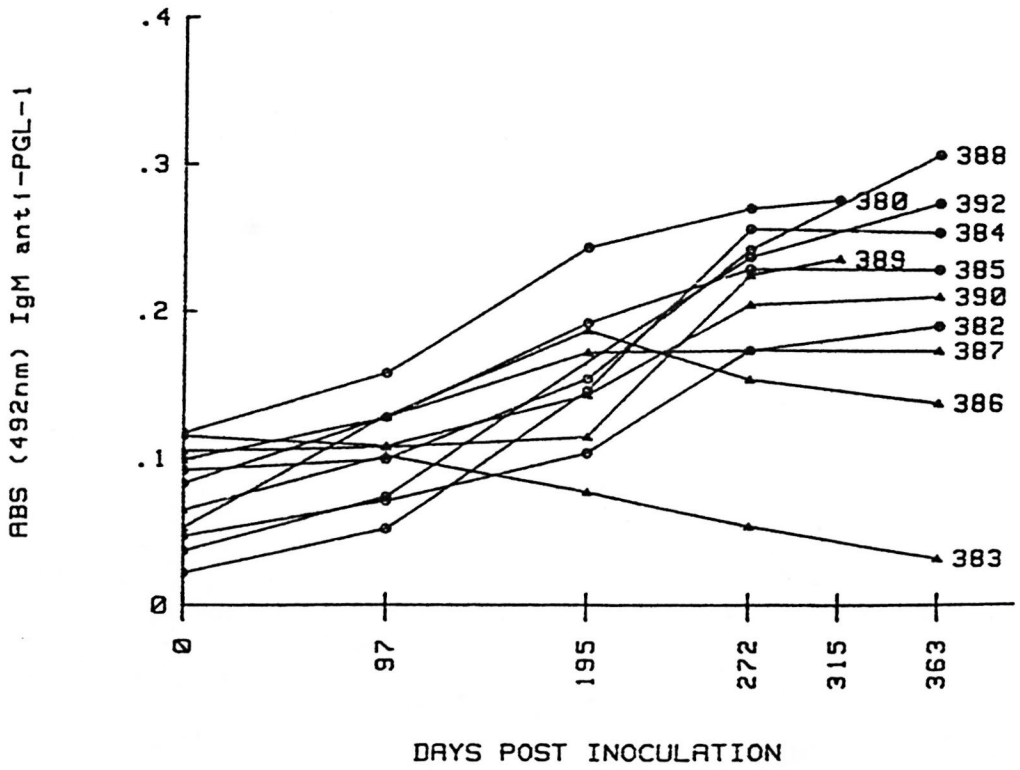

FIG. 3. Kinetics of IgM anti-PGL-I.

low absorbance values. These findings suggest that the ability to control infection with *M. leprae* in both armadillos and leprosy patients may be associated with their ability to mount an IgG antibody response to PGL-I.

While data reflecting antibody responses to PGL-I, including ours, are presented as absorbance values, these values do not directly reflect absolute values for IgG or IgM antibodies. Antibodies to *M. leprae* are not expected to be protective in leprosy since *M. leprae* is an intracellular organism, but production of IgG anti-PGL-I antibodies might be a reflection of more competent T-cell function than the production of IgM antibodies alone. Cho, *et al.* [2] were the first to suggest that the high levels and the persistence of IgM antibody to PGL-I in lepromatous patients may be due to a lack of T-helper cell-mediated switch from IgM to IgG antibody. Levis, *et al.* [9] and Koster, *et al.* [8] have proposed that a significant IgG anti-PGL-I response among paucibacillary leprosy patients may be due to successful T-cell help for an IgM to IgG switch. Thus, there may be an association between elevated IgG anti-PGL-I responses and the T-cell immunological recognition of *M. leprae.*

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

SUMMARY

The kinetics of antibody responses of *Mycobacterium leprae*-infected armadillos to phenolic glycolipid-I (PGL-I) were studied by means of ELISA. The levels of both IgG

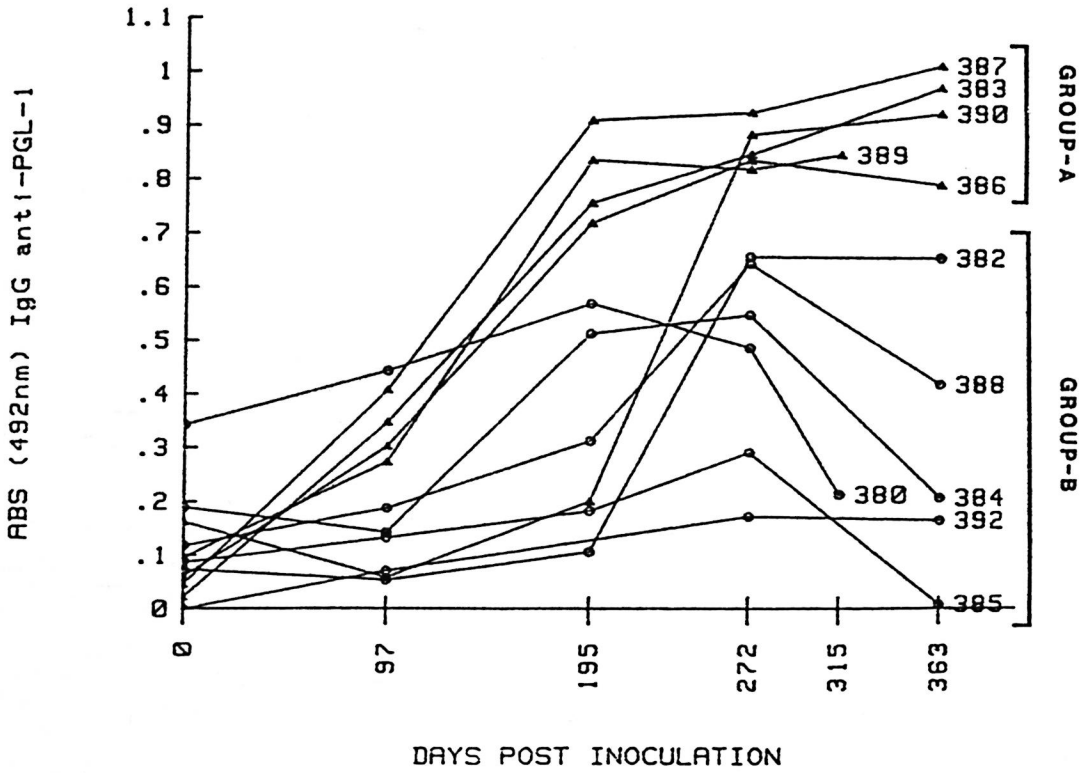

FIG. 4. Kinetics of IgG anti-PGL-I. Animals in group A showed relative resistance to *M. leprae* infection and were still alive 2½ yr postinoculation; group B animals were more susceptible to *M. Leprae* infection, had widely disseminated disease, and were sacrificed within 1 yr of inoculation.

and IgM antibodies to PGL-I increased with time. Some animals were less susceptible to disseminations of *M. leprae* infection and lived longer than others. These animals had high absorbance values (>0.7) for IgG anti-PGL-I compared to more susceptible armadillos that had lower absorbance values for IgG anti-PGL-I.

RESUMEN

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

RÉSUMÉ

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

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