Partial Characterization of Antigens from *M. leprae* Evoking IgG and IgM Antibodies in Armadillos[1,2]

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The nine-banded armadillo (*Dasypus novemcinctus*) is highly susceptible to infection with *Mycobacterium leprae* and has become an important animal model in the study of leprosy ([6]). Results describing serologic responses to *M. leprae* in armadillos have implicated several distinct antigenic components of the bacillus as major immunogens. These include, antigen 7 ([4]), cell wall polysaccharides ([1]), and the unique glycolipid molecule, phenolic glycolipid-1 (PGL-I) ([12]).

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

MATERIALS AND METHODS

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

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

Rabbit anti-armadillo IgG (γ-chain-specific). Fractions enriched for armadillo IgG by protein-A chromatography were reduced and heated (100°C for 3 min) in 0.1% SDS and 1% 2-mercaptoethanol and separated by SDS-PAGE ([8]) consisting of 4% stacking gel and 12% separating gel. One lane of the gel was stained with Coomassie brilliant blue R250 to locate the position of the immunoglobulin (Ig) heavy and light chains. The portion of the remaining unstained gel containing the heavy chains was removed, minced, and suspended in PBS. This suspension was injected subcutaneously into the foot pads and multiple sites on the backs of rabbits. The animals were given a booster injection 3 weeks later. The immune sera were collected 30 days after the primary injection.

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

[1] Wayne M. Meyers, M.D., Ph.D., kindly served as Editor in regard to the submission, review, revision, and acceptance of this manuscript.

[2] Received for publication on 19 February 1987; accepted for publication in revised form on 28 January 1988.

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method described by the manufacturer. The rabbit anti-armadillo γ-chain-specific antibodies were eluted from the column with 0.1 M glycine, pH 2.8, and dialyzed against PBS.

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

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

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

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

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

^{125}I-ConA binding of the *M. leprae* extracts. Concanavalin-A (ConA; Sigma Chemical Co., St. Louis, Missouri, U.S.A.) was radioiodinated by the Chloramine-T method ([3]). Free iodine was removed by gel filtration on P-6DG (Bio-Rad). *M. leprae* extracts were separated by SDS-PAGE and then electrophoretically transferred from the gel onto NCP. The NCP was incubated in a 1% BSA/PBS solution for 30 min at room temperature, and then incubated in 40 ml of ^{125}I-ConA (1×10^6 cpm/ml) in 1% BSA/PBS for 1 hr at room temperature. The NCP was washed to remove unbound ^{125}I-ConA, dried, and exposed to Kodak Ortho G film (Eastman Kodak Co., Rochester, New York, U.S.A.). Exposure of the NCP was at −70°C for 12 hr in an x-ray cassette fitted with a Coronex MRF 32 clear base intensifying screen (Dupont Co., Newton, Connecticut, U.S.A.).

RESULTS

Characterization of rabbit anti-armadillo γ-chain-specific serum. Armadillo serum proteins were fractionated on Sephacryl S-200, resolving the material into three distinct peaks (Fig. 1A). Peak II from the Sephacryl column was applied to a protein-A Sepharose CL-4B column and unbound material eluted with PBS, pH 7.2 (peak a, Fig. 1B). Protein-A bound material was eluted by the addition of low pH buffer (peak b),

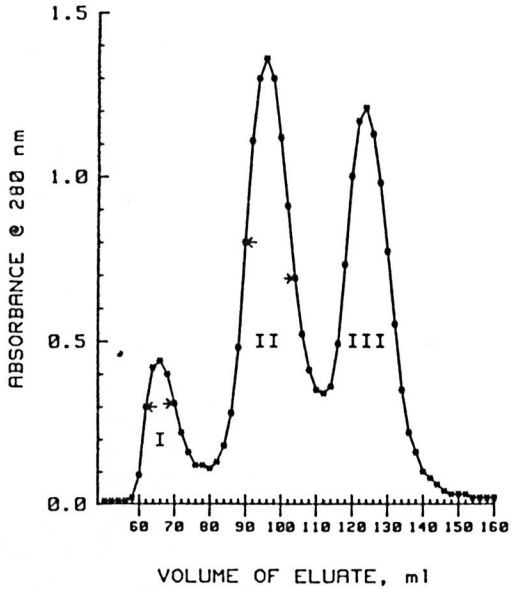

FIG. 1A. Sephacryl S-200 gel filtration of pooled normal armadillo serum. Arrows indicate fractions pooled for analysis.

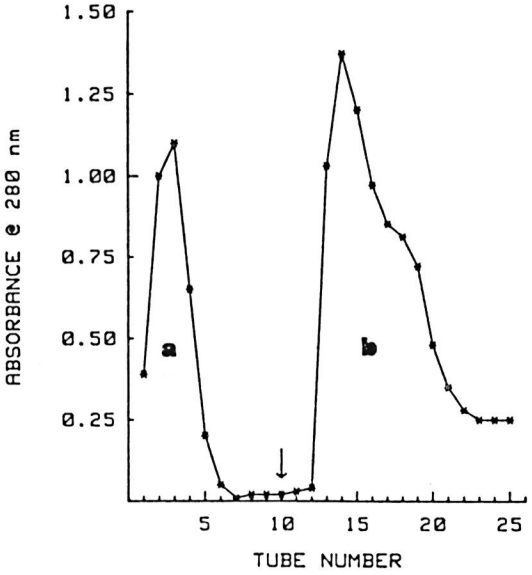

FIG. 1B. Protein-A affinity chromatography of Peak II from Sephacryl S-200 column (Fig. 1A). Arrow indicates addition of low pH buffer.

and this material was analyzed by immunoelectrophoresis. A single immunoprecipitate band was observed when a sample of peak b was electrophoresed in agarose and then allowed to react with rabbit anti-whole armadillo serum (data not shown). The immune precipitate formed was localized in the zone characteristic for human IgG. SDS-PAGE analysis of peak b from the protein-A column (armadillo IgG) (Fig. 2, lane B) and human IgG (Fig. 2, lane C) showed identical patterns of migration for the reduced heavy chain molecule. In contrast, the major species of armadillo light chain (Fig. 2, lane B) was slightly higher in molecular weight (M_r = 28,000) as compared to those of human (M_r = 25,000) (Fig. 2, lane C). Rabbit antibodies reactive with the 51-kDa protein were purified by affinity chromatography using purified armadillo IgG as immunosorbent. Immunoreactivity of the purified antibodies located a single band at 51 kDa present in both purified armadillo IgG (Fig. 3, lane B) and whole armadillo sera (Fig. 3, lane C).

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

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

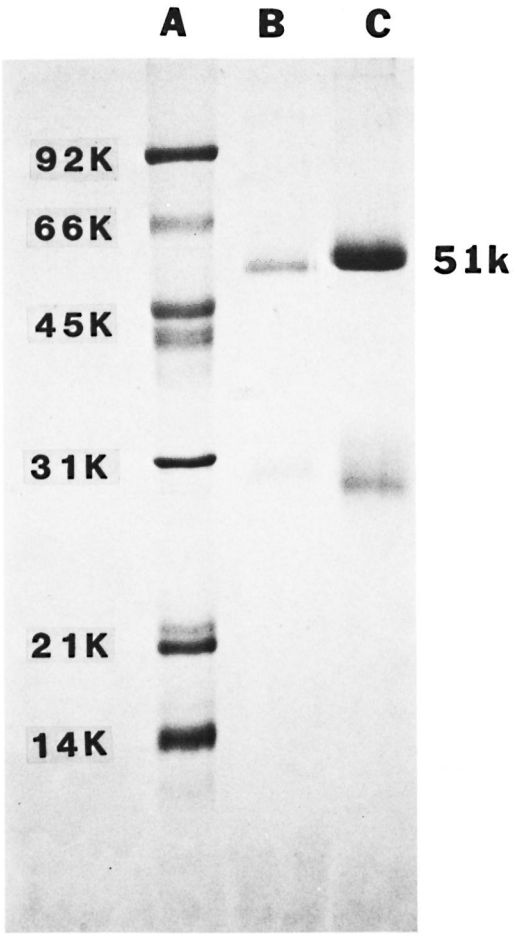

FIG. 2. Analysis of armadillo and human IgG by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were stained with Coomassie blue. Lane A = low molecular weight protein standards: phosphorylase b (92 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor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

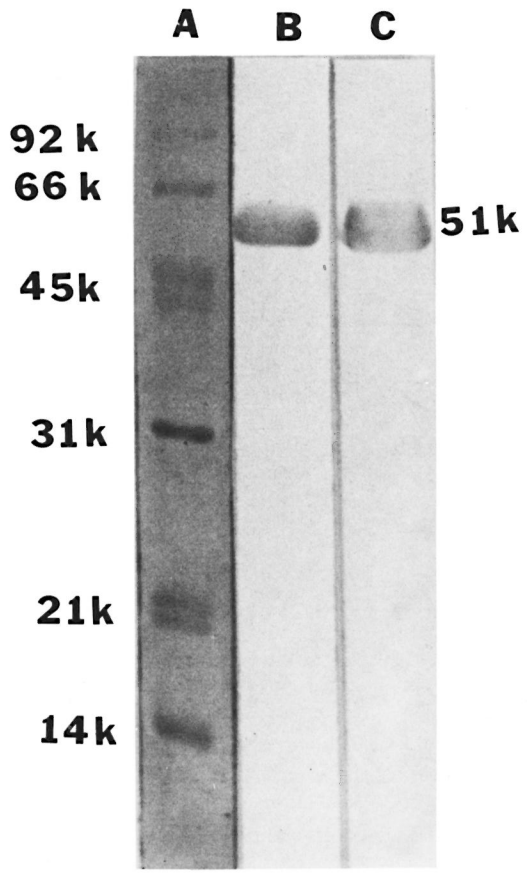

FIG. 3. Immunoblot of rabbit anti-armadillo γ-chain-specific sera. Armadillo whole serum at 1:100 dilution and purified armadillo IgG (0.9 μg) were analyzed by immunoblotting. Nitrocellulose paper (NCP) was incubated with rabbit anti-armadillo γ-chain sera at 1:1000 dilution, washed, and incubated with peroxidase conjugated goat anti-rabbit immunoglobulins at 1:1000 dilution. NCP was washed and treated with dye-developing reagent (H_2O_2, 4-chloro-1 naphthol). Lane A = low molecular weight protein standards (see Fig. 2 legend); lane B = purified armadillo IgG; lane C = armadillo whole serum.

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

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

DISCUSSION

The mechanism by which *M. leprae* eludes the host defense system to cause disease is unknown. Since protection against disease

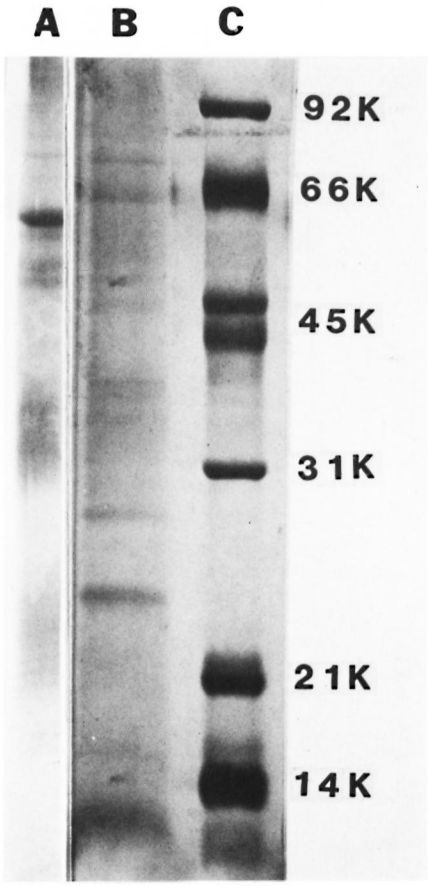

FIG. 4. Staining characteristics of *M. leprae* extracts. Soluble extracts were stained for detection of proteins and carbohydrates. Lane A = detection of components containing carbohydrate moieties by silver stain modified from the Morrisey procedure ([10]); lane B = detection of components containing proteins by Coomassie brilliant blue staining of the gel followed by silver staining; lane C = low molecular weight markers.

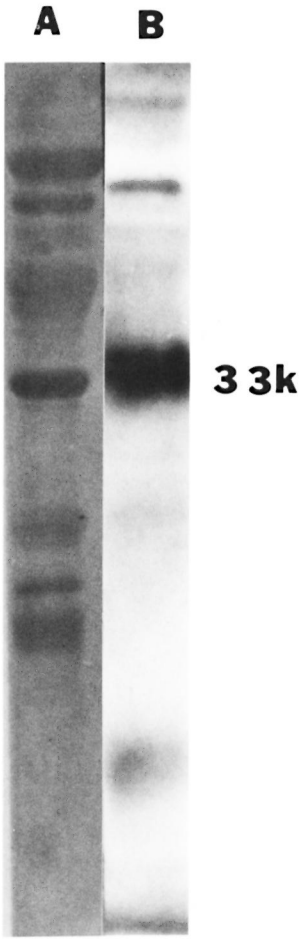

FIG. 5. Identification of antigenic components of *M. leprae* reacting with ^{125}I-ConA. Supernatant extracts of *M. leprae* were fractionated by SDS-PAGE and electrophoretically blotted onto NCP. NCP was incubated with ^{125}I-ConA and subsequently autoradiographed.

is associated with an appropriate host immune response to *M. leprae,* delineation of the antigenic constitution of *M. leprae* is essential to the understanding of this process. This study was undertaken to characterize the antigenic components of *M. leprae* which evoked IgG or IgM antibody responses in normal and heavily infected armadillos. An antiserum specific to armadillo IgG (γ-chain) was prepared to identify the antigenic components of *M. leprae* which elicited IgG antibody responses in armadillos. IgM responses were detected using an anti-human μ-chain-specific reagent which has been shown to crossreact with armadillo IgM ([12]).

The IgG responses of heavily infected armadillos were directed at approximately 10 distinct bands with molecular weights from 33 kDa to 90 kDa and two additional bands at 12 kDa and 22 kDa. Chakrabarty, *et al.* ([1]) also reported immunoreactivity in heavily infected armadillos to the 12 kDa and 22 kDa antigen of *M. leprae,* but observed only four other major immunoreactive bands with molecular weights from 33 kDa to 67 kDa. It is possible that some of the additional immunoreactive components observed in our studies were degrative

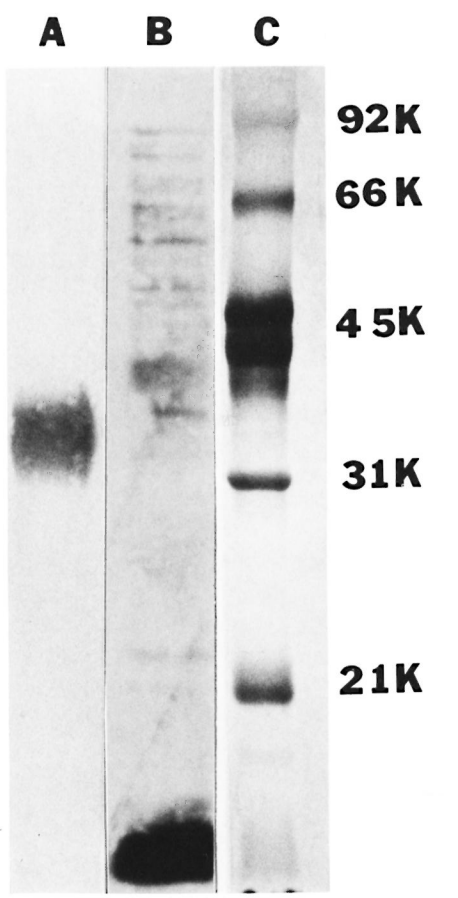

FIG. 6A. Demonstration of antigenic components of *M. leprae* eliciting IgG and IgM antibody responses. Soluble extracts of *M. leprae* were separated by SDS-PAGE and electrophoretically blotted onto NCP. NCP was incubated with infected armadillo serum (1:50 dilution), washed, and treated with class-specific antisera (rabbit anti-armadillo γ-chain at 1:1000 dilution; rabbit anti-human μ-chain at 1:50 dilution). The strips were washed and treated with peroxidase-conjugated goat anti-rabbit immunoglobulin (IgA + IgM + IgG) at 1:1000 dilution. Visualization of antigen-bound antibody proceeded as explained in Figure 3. Lane A = reaction with rabbit anti-human μ-chain-specific antibody; lane B = reaction with rabbit anti-armadillo γ-chain-specific antibody; lane C = low molecular weight markers.

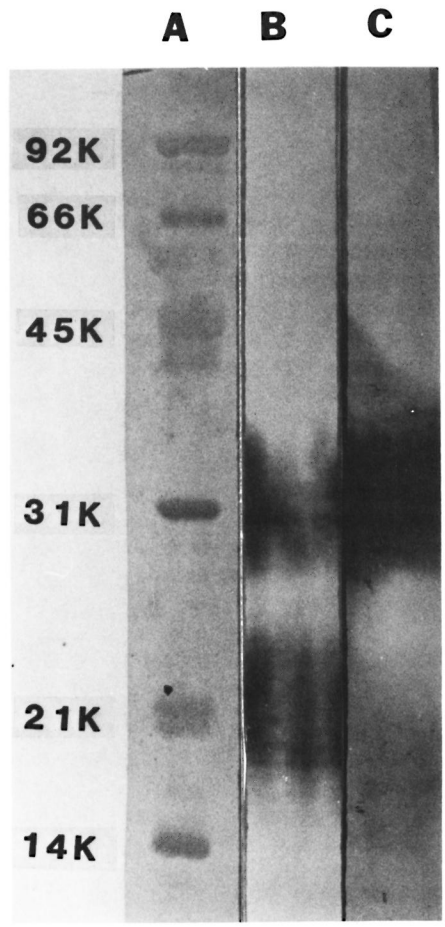

FIG. 6B. Immunoblot to demonstrate IgG and IgM antibody responses of normal armadillos to *M. leprae.* (Technical procedure is same as in Fig. 6A.) Lane A = low molecular weight markers; lane B = reaction with rabbit anti-armadillo γ-chain-specific antibody; lane C = reaction with rabbit anti-human μ-chain-specific antibody.

components of related molecules reported by Chakrabarty, *et al.* [1]. This seems unlikely, however, because the *M. leprae* extract used in our study was prepared under the same conditions as that of Chakrabarty, *et al.* [1]. In addition, we observed bands of immunoreactivity at the region of 70 kDa to 90 kDa which were not reported in their studies. One explanation for the differences in antibody responses seen between the two studies is the likely individual variations in antibody responses among the highly outbred population of armadillos. Alternatively, recognition of larger numbers of immunoreactive bands could be due to a potential increase in sensitivity and specificity of our assay using isotype-specific reagents, as opposed to radiolabeled protein-A.

Furthermore, the above pattern of IgG immunoreactivity may reflect the relative immunodominance or accessibility of these proteins in the intact bacillus, or their rel-

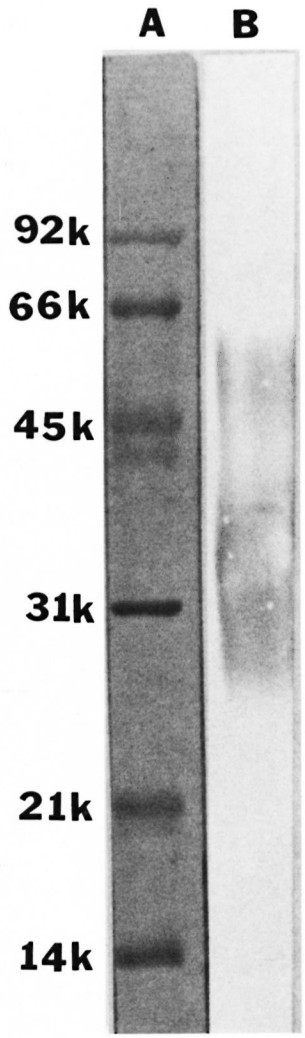

FIG. 7. Immunoblot to demonstrate IgM antibody response of infected armadillos to lipoarabinomannan (LAM). LAM (80 μg/ml) was separated by SDS-PAGE and electrophoretically blotted onto NCP. (Technical procedure is same as in Fig. 6A.) Lane A = low molecular weight markers; lane B = reaction with rabbit anti-human μ-chain-specific antibody.

ative concentration in the bacterial cell sonicate. Similarities between human and armadillo antibody responses have been reported by noting immunoreactivity to *M. leprae* components with molecular weights of 12, 22, 28, 33, 36, 41, and 86 kDa ([1, 7]). Immunoblot analysis of multibacillary patients reported by Chakrabarty, *et al.* ([1]) predominantly detected two bands of immunoreactivity with molecular weights of 12 kDa and 33 kDa, whereas the study performed by Klatser, *et al.* ([7]) reported as many as six bands of immunoreactivity with molecular weights of 12, 22, 28, 36, 41, and 86 kDa.

Comparison of immunoblot analyses of armadillo IgG responses to *M. leprae* components with that of humans indicates that *M. leprae*-infected armadillos generally recognized and produced antibodies to a larger number of *M. leprae* components than multibacillary patients. For example, analysis of sera from *M. leprae*-infected armadillos indicated that, in addition to the above immunoreactive components recognized by human sera, armadillo sera reacted to a group of *M. leprae* components with molecular weights ranging from 45 kDa to 90 kDa and, among these, only the 86 kDa component of *M. leprae* was recognized by human sera ([7]). Our results support earlier findings that armadillos produce antibody to a great diversity of *M. leprae* antigens as opposed to the previously reported limited repertoire produced by Hansen's disease (HD) patients. Differences in antibody profiles between experimental *M. leprae*-infected armadillos and humans afflicted with HD could be due to the effect of chemotherapy of patients as suggested by Klatser, *et al.* ([7]), or as a result of immune recognition of various antigens governed by immune response genes.

The predominant IgM antibody responses of the majority of infected armadillos were to a component which migrated as a broad diffuse band with a molecular weight of approximately 28–33 kDa. Staining characteristics using a modified silver stain for carbohydrates and the ability of the 33 kDa antigen to bind ^{125}I-ConA suggested that this molecule is composed of carbohydrates containing mannose or glucose residues. Additionally, when we analyzed the IgG and IgM antibody responses of an infected armadillo to the highly cross-reactive mycobacterial antigen LAM ([3]) derived from *M. tuberculosis,* IgM activity was observed only to two broad diffuse bands at the regions of 28–33 kDa and 50 kDa. Comparison of the SDS-PAGE migration patterns and antibody binding characteristics of the 33 kDa material with that of purified *M. tuberculosis* LAM ([5]) suggested that

the two components were chemically and immunologically related.

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

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

SUMMARY

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

RESUMEN

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

RÉSUMÉ

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

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

REFERENCES

1. Chakrabarty, A.K., Maire, M. A. and Lambert, P. H. SDS-PAGE analysis of *M. leprae* protein antigens reacting with antibodies from sera from lepromatous patients and infected armadillos. Clin. Exp. Immun. **49** (1982) 523–531.
2. Ellner, J. J. and Daniel, T. M. Immunosuppression by mycobacterial arabinomannan. Clin. Exp. Immunol. **35** (1979) 250–257.
3. Greenwood, F. C., Hunter, W. M. and Clover, J. S. The preparation of ^{131}I-labeled human growth

hormone of high specific radioactivity. Biochem. J. **89** (1963) 114–123.

4. HARBOE, M., CLOSS, O., REES, R. J. W. and WALSH, G. P. Formation of antibody against *Mycobacterium leprae* antigen 7 in armadillos. J. Med. Microbiol. **11** (1978) 525–535.
5. HUNTER, S. W., GAYLORD, H. and BRENNAN, P. J. Structure and antigenicity of the phosphorylated lipopolysaccharide antigens from the leprosy and tubercule bacilli. J. Biol. Chem. **261** (1986) 12345–12351.
6. KIRCHHEIMER, W. F. and STORRS, E. E. Attempts to establish the armadillo (*Dasypus novemcinctus* Linn.) as a model for the study of leprosy. Int. J. Lepr. **39** (1971) 693–702.
7. KLATSER, P. R., VAN RENS, M. M. and EGGELET, T. A. Immunochemical characterization of *Mycobacterium leprae* antigens by SDS-polyacrylamide gel electrophoresis immunoperoxidase technique (SGIP) using patients' sera. Clin. Exp. Immunol. **56** (1984) 537–544.
8. LAEMMLI, U. K. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature **227** (1970) 680–689.
9. LOWRY, O. H., ROSEBROUGH, N. S., FARR, A. M. and RANDALL, R. J. Protein measurement with the folin phenol reagent. J. Biol. Chem. **193** (1970) 265–272.
10. MORRISEY, J. H. Silver stain for protein in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. J. Biol. Chem. Anal. Biochem. **117** (1981) 307–310.
11. TOWBIN, H. M., STAEHELIN, T. and GORDON, J. Electrophoretic transfer of proteins from poly-acrylamide gels to nitrocellulse sheet; procedure and applications. Proc. Natl. Acad. Sci. U.S.A **76** (1979) 4350–4354.
12. TRUMAN, R. W., SHANNON, E. J., HAGSTAD, H. V., HUGH-JONES, M. E., WOLFF, A. and HASTINGS, R. C. Evaluation of the origin of *Mycobacterium leprae* infections in the wild armadillo, *Dasypus novemcinctus*. Am. J. Trop. Med. Hyg. **35** (1986) 588–593.