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Ability of the Phenolic Glycolipid-I Antigen of *M. leprae* to Elicit a Positive Mitsuda Response in the Armadillo (*Dasypus novemcinctus*)[1,2]

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Recently, a chemically defined antigen called phenolic glycolipid-I (PGL-I) has been isolated from *Mycobacterium leprae* ([5]). Antibodies to this glycolipid have been demonstrated in leprosy patients and normal individuals exposed to the disease using enzyme-linked immunosorbant assay (ELISA) techniques, and they appear to be immunologically specific to *M. leprae* ([3, 6, 14]). Although much work has been done on the antibody response to PGL-I in patients and in normal populations ([1, 3, 6, 11, 14]), studies on its activity in delayed hypersensitivity or cell-mediated immune (CMI) responses are few ([2, 8]). In this report we have studied the ability of this purified antigenic determinant of *M. leprae* to produce a positive lepromin (positive Mitsuda) response in leprosy-resistant armadillos.

MATERIAL AND METHODS

Purified PGL-I was obtained from Dr. Patrick Brennan's laboratory (Department of Microbiology, Colorado State University, Fort Collins, Colorado, U.S.A.), and a suspension in 0.85% w/v saline was prepared by sonicating 1.9 mg of PGL-I in 1.9 ml of saline for 30 sec using a Branson sonifier cell-disruptor at 100 watts power setting. The suspension was then diluted with saline so that 0.1 ml of the suspension contained 100 μg of PGL-I. Incomplete Freund's adjuvant was obtained from Difco Laboratories (Detroit, Michigan, U.S.A.). Lepromin was prepared from subcutaneous nodules of *M. leprae*-infected armadillos and contained 1.6×10^8 integral *M. leprae* per ml in 0.5% v/v phenol in normal saline. One tenth ml was injected intradermally.

Two groups of armadillos were used in this experiment. One group of three had been previously infected intravenously with 10^8 *M. leprae,* had been found resistant to the disease, and was known to be lepromin positive. The other group of three consisted of two armadillos previously infected intravenously with 10^8 *M. leprae* which had not developed the disease after having been followed for over 4 years. These animals were lepromin negative. The third animal in the group was an uninfected, lepromin-negative armadillo.

All six of the armadillos were injected intradermally in the abdominal skin with 100 μg of PGL-I suspended in 0.1 ml of normal saline. In addition, the three lepromin-negative armadillos and two of the three lepromin-positive armadillos were injected intradermally at a separate site with 0.1 ml of Freund's incomplete adjuvant. Tattoo marks were placed around the injected sites. At 21 days, the diameters of induration of the skin reactions were measured in two directions perpendicular to each other and the mean of the two readings was recorded.

All skin-test sites were biopsied using a 4-mm punch. The biopsies were bisected. Representative pieces were taken from the middle of the specimen from one lepromin-positive and one lepromin-negative animal tested with PGL-I, and were processed for electron-microscopic study. All 11 specimens were processed for light-microscopic examination. They were fixed in 10% buffered Formalin for 48 hr, and then processed for paraffin sections. Five μm sections were cut, stained with hematoxylin and eosin and

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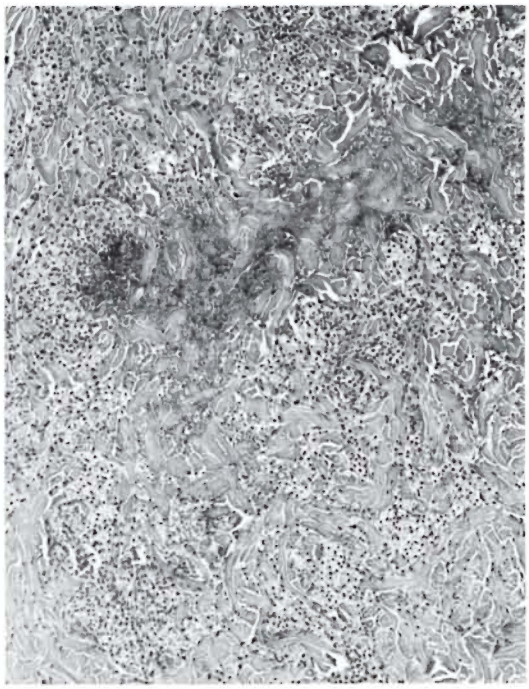

FIG. 1. Photomicrograph of a positive skin reaction to PGL-I showing many large collections of epithelioid cells and an area of necrosis (H&E ×140).

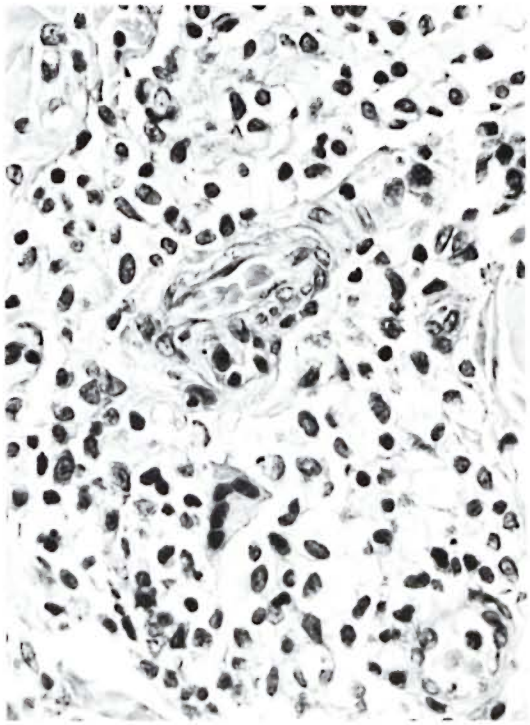

FIG. 2. Photomicrograph of a positive skin reaction to PGL-I showing a poorly formed giant cell among many epithelioid cells, a few lymphocytes, and an occasional neutrophil (H&E ×450).

an acid-fast stain according to a modified Fite's method ([7]), and then examined. The tissues obtained for electron-microscopic examination were further subdivided into 1-mm cubes fixed immediately in 4% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 at 4°C for 2 hr. They were then rinsed in several changes of buffer containing 0.2 M sucrose for 24 hr, were dehydrated in a graded series of ethanol followed by immersion in propylene oxide, and were embedded in Spurr resin for making ultrathin sections. First, 1 μm sections were made, stained with toluidene blue, and screened. Representative blocks containing the granulomas were selected, ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and examined using a Philips 410 electron microscope.

RESULTS

Lepromin-positive armadillos. The lepromin reaction of the three animals at 21 days measured 8 mm, 10 mm, and 14 mm. The histopathological appearances of all three tests were very similar. There were collections of epithelioid cells, a few macrophages, neutrophils, and some lymphocytes scattered in a wide area in the dermis. Several focal areas of necrosis were also present. Many of the epithelioid cells had a vacuolated cytoplasm. Lymphocytes were fewer than epithelioid cells. An acid-fast stain showed bacilli in the areas of necrosis and inside a few macrophages immediately surrounding the necrotic areas.

The diameters of the skin reactions in the three lepromin-positive animals tested with PGL-I measured at 21 days were 3 mm, 3 mm, and 7 mm. The histopathology of all three biopsies showed a more-or-less similar appearance. There were large collections of inflammatory cells composed of a mixture of epithelioid cells, lymphocytes, and occasional neutrophils surrounding an area of necrosis (Fig. 1). In some areas a few poorly formed giant cells were seen (Fig. 2). The epithelioid cells were the predominant component of the granuloma, and the lymphocytes were less prominent. Acid-fast staining showed no organisms. The elec-

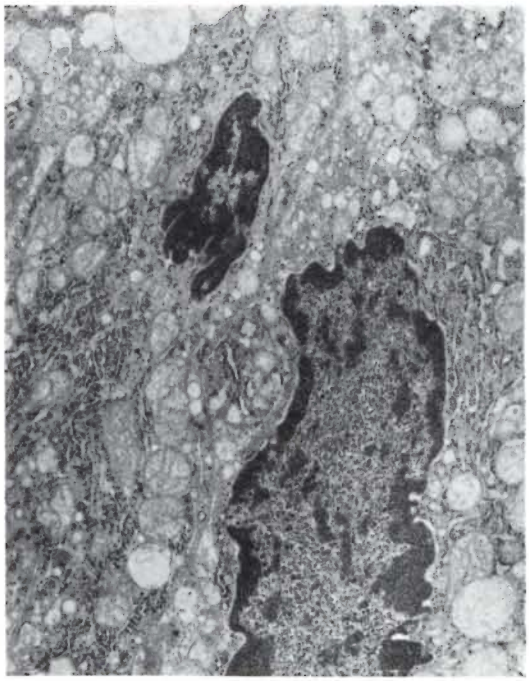

FIG. 3. Electron micrograph of a positive skin reaction to PGL-I showing activated macrophages containing numerous mitochondria, markedly increased rough endoplasmic reticulum, and a few vacuoles (×7600).

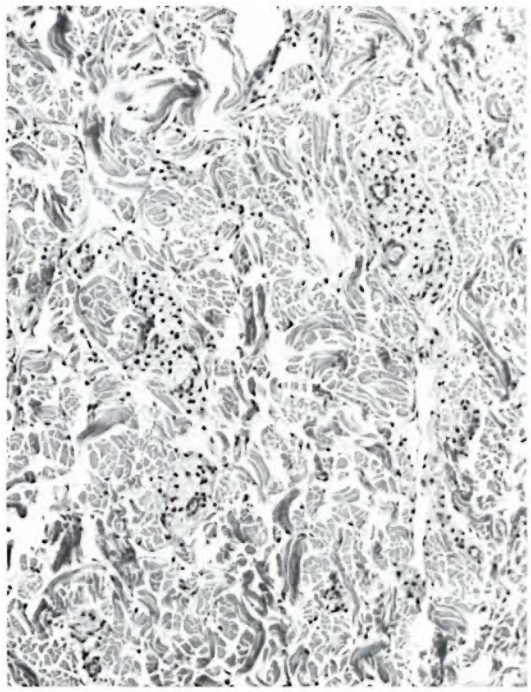

FIG. 4. Photomicrograph of a negative skin reaction to PGL-I. Small focal collections of macrophages are seen around blood vessels (H&E ×140).

tron-microscopic examination confirmed the above findings. A large majority of the cells were epithelioid cells with large and convoluted nuclei with several indentations (Fig. 3). There was also a marked increased in the endoplasmic reticulum and numerous mitochondria. A few small intracellular vacuoles containing amorphous material were also present.

The two animals tested with Freund's incomplete adjuvant showed indurations at the test sites measuring 25 mm and 13 mm. Histopathological examination showed sheets of macrophages, most of them with a vacuolated cytoplasm and some with a pink granular cytoplasm. They were admixed with some lymphocytes.

Lepromin-negative armadillos. The lepromin reactions of the three armadillos were clinically negative, and the histopathological appearances were almost identical. The inflammatory infiltrate was scanty, and was composed almost entirely of very small collections of foamy macrophages distributed in a wide area in the dermis. Acid-fast staining showed clumps of bacilli inside the foam cells.

The skin reactions in the three lepromin-negative animals tested with PGL-I were all macroscopically negative. The test sites were identified by the tattoo marks since there were no gross reactions at the test sites. The histopathological appearance of the test sites showed the structures of normal skin which, on careful examination, showed a few small clumps of inflammatory cells around dermal capillaries (Fig. 4). Almost all of the inflammatory cells were macrophages with a clear cytoplasm (Fig. 5). The paraffin blocks of the biopsies had to be properly oriented to detect these small macrophage clumps. Acid-fast staining showed no bacilli. The electron-microscopic examination of one of the biopsy specimens showed mainly macrophages with much of the cytoplasm containing many small and large vacuoles (Fig. 6). The vacuoles contained an amorphous material, and between these vacuoles a few mitochondria were identified.

The skin reactions at the sites of the three armadillos injected with Freund's incomplete adjuvant measured 23 mm, 28 mm,

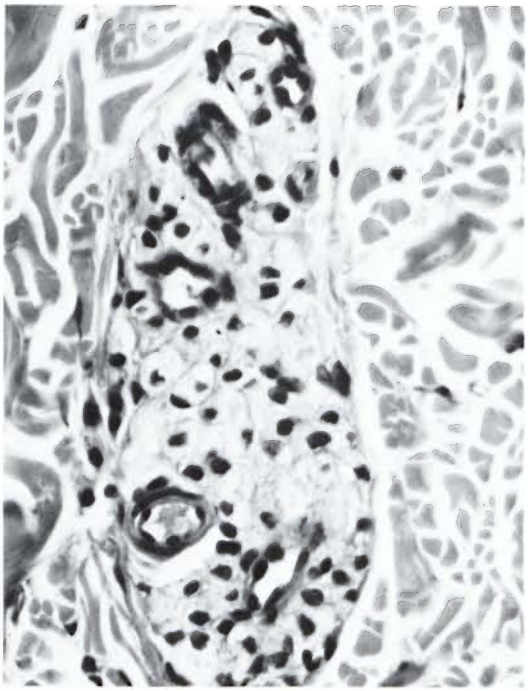

FIG. 5. High-power photomicrograph of Figure 4. Note macrophages having a clear cytoplasm (H&E ×450).

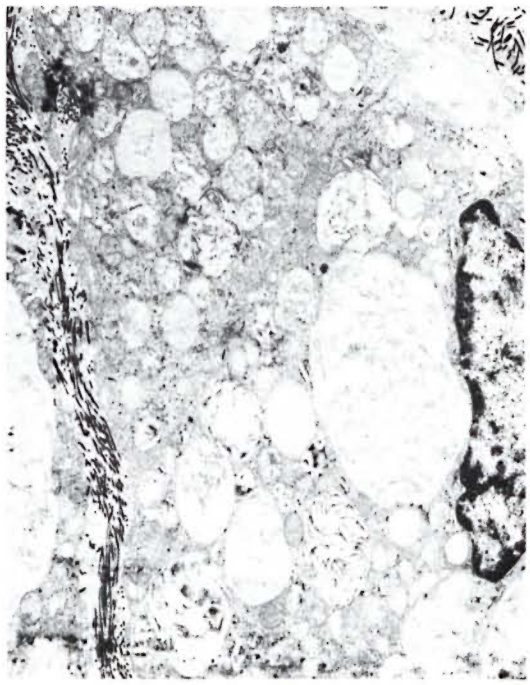

FIG. 6. Electron micrograph of a negative skin reaction to PGL-I showing macrophages containing numerous phagosomes, a few mitochondria, and scanty rough endoplasmic reticulum. Phagosomes contain amorphous granular material (×13, 800).

and 31 mm. Histopathologically, these nodular indurations were uniformly composed of large sheets of macrophages, most of which had a vacuolated cytoplasm. There were also very unevenly distributed lymphocytes.

DISCUSSION

In our experience, the classical delayed-type hypersensitivity (DTH) reaction at 24 hr and 48 hr to autoclaved suspensions of whole *M. leprae* is difficult to differentiate from a nonspecific inflammatory reaction in the armadillo skin (unpublished observations). The 48-hr lepromin reaction to integral *M. leprae* is known to be highly variable and to correlate poorly with the clinical forms of leprosy (9). For these reasons, we chose to study only the late lepromin reaction.

The three lepromin-positive armadillos gave a clinically demonstrable reaction at the skin sites injected with 100 μg of PGL-I in 0.1 ml of saline measuring 3 mm, 3 mm, and 7 mm; whereas the measurements of the lepromin reactions were 8 mm, 10 mm, and 14 mm in the respective animals. Histologically, the lepromin test site showed several focal areas of necrosis along with many collections of epithelioid cells, lymphocytes, and a few neutrophils occupying a large area of the dermis. Obviously, the lepromin reaction was two to three times larger in diameter than the reaction elicited by PGL-I. However, the histopathological responses to both antigens were similar. The cellular reactions elicited by PGL-I mimicked those of lepromin and were, indeed, hypersensitivity granulomas. The electron-microscopic observations showed activated macrophages maturing to epithelioid cells, thus confirming the light-microscope findings.

The histopathologic features of the lepromin reactions and the responses to PGL-I in the lepromin-negative animals were almost alike, except that the macrophages in the lepromin reactions were distributed more widely in the dermis and there were clumps of acid-fast bacilli inside them. Clinically, no reactions were evident at the test

sites to either antigen. The electron-microscopic study of the reaction to PGL-I confirmed the foamy degeneration of the macrophages.

A positive Mitsuda test is now widely recognized as a manifestation of delayed hypersensitivity with the formation of an immune granuloma at the site of injection around persisting antigens from the killed *M. leprae* ([12]). Further, the Mitsuda test is used by clinicians all over the world to assess the *M. leprae*-specific, cell-mediated immune (CMI) status of their patients ([9]). In an intense reaction, the granuloma is also often associated with areas of necrosis ([13]). The response of PGL-I obtained in lepromin-positive armadillos is almost identical to that of lepromin, thus demonstrating that purified PGL-I is capable of eliciting an immune granuloma in these resistant armadillos.

Brett, *et al.* reported that PGL-I failed to elicit the classical delayed-type hypersensitivity response at 24 hr and 48 hr in mice immunized with *M. leprae* and that only a nonspecific inflammatory response was present ([2]). Careful examination of the photomicrographs published in their paper, illustrating the 48-hr response to 50 μg of PGL-I in mice immunized previously with 10^9 *M. leprae* together with the positive and the negative controls, confirms the difficulty in differentiating between a delayed-type hypersensitivity response and a nonspecific inflammatory response.

Incomplete Freund's adjuvant produced a granulomatous response consisting of mainly macrophages and lymphocytes in both lepromin-positive and lepromin-negative animals. The fact that the positive responses to PGL-I were present selectively in lepromin-positive armadillos and that there was necrosis in the midst of the granuloma in one animal strongly suggest that the skin-test responses to PGL-I ([4]) are not nonspecific responses to lipid material or foreign body granulomas.

Lepromin derived from the armadillo (lepromin A) has almost replaced lepromin derived from human patients (lepromin H) because of the reduction in the numbers of nodular lepromatous patients worldwide and the emergent nonavailability of a regular supply of lepromin H. However much the organisms isolated from the armadillos are purified, it is almost impossible to remove all armadillo tissue from lepromin A. It would be worth investigating further to find out if the purified PGL-I or PGL-I combined with an antigenic protein isolated from *M. leprae* can substitute for whole bacilli in the Mitsuda test.

Brett, *et al.* ([2]) showed no evidence that PGL-I is active in delayed-type hypersensitivity or cell-mediated immune systems in mice. Mehra, *et al.* ([8]) and more recently Modlin, *et al.* ([10]), on the other hand, have presented data that PGL-I is responsible for the suppression of proliferation by human peripheral blood mononuclear cells in response to concavalin A and is therefore active, at least indirectly, in delayed-type hypersensitivity and cell-mediated immunity. To the extent that positive delayed-hypersensitivity granulomas are dependent on T-lymphocyte immunoreactivity and to the extent that purified PGL-I in a dose of 100 μg does not contain biologically significant quantities of other antigens of *M. leprae,* delayed-type hypersensitivity and cell-mediated immune responses to *M. leprae* in armadillos.

It would be interesting to conduct a similar study in a group of lepromin-positive and lepromin-negative leprosy patients to find out if the same results can be obtained in humans.

SUMMARY

Three lepromin-positive armadillos and three lepromin-negative armadillos were tested intradermally with 100 μg of phenolic glycolipid-I (PGL-I) in 0.1 ml of normal saline. Positive delayed-hypersensitivity granulomas at 21 days in the lepromin-positive animals and negative responses in the lepromin-negative animals were obtained. These observations suggest that purified PGL-I is capable of eliciting cell-mediated immune or delayed-hypersensitivity responses in animals sensitized to *Mycobacterium leprae.*

RESUMEN

Tres armadillos lepromino-positivos y 3 armadillos lepromino-negativos se probaron intradérmicamente con 100 μg de glicolípido fenólico-I (GLF-I) en 0.1 ml de solución salina normal. A los 21 días se obtuvieron

granulomas de hipersensibilidad retardada en los animales lepromino-positivos y respuestas negativas en los animales lepromino negativos. Estas observaciones sugieren que el GLF-I purificado es capaz de inducir respuestas de inmunidad celular o de hipersensibilidad retardada en animales sensibilizados al *Mycobacterium leprae.*

RÉSUMÉ

On a testé trois tatous positifs à la lépromine, et trois tatous négatifs, par voie intradermique, avec 100 μg de l'antigène phénoglycolipidique-I (PGL-I) dans 0,1 ml de sérum physiologique normal. On a obtenu ainsi des granulômes d'hypersensibilité retardée positifs au 21ème jour chez les animaux positifs, mais des réponses négatives chez les animaux négatifs à la lépromine. Ces observations font penser que l'antigène purifié PGL-I peut induire des réponses d'immunité à médiation cellulaire ou d'hypersensibilité retardée chez les animaux sensibilisés à *Mycobacterium leprae.*

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