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Ganglioside Patterns in Normal and Lepromatous Armadillo Tissues[1]

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Gangliosides, also referred to as glycosphingolipids, are unique glycolipids present in the plasma membranes of mammalian cells and are particularly abundant in neural tissues. Gangliosides have attracted considerable attention in recent years as participants in a variety of cellular functions. A number of investigations have implicated these complex carbohydrates in cell differentiation and growth, as surface membrane receptors, and as glycolipid antigens having a role in regulating immune responses ([4]).

Gangliosides, as the most specific lipids of neurons, very likely have a role in neurofunction ([7]). It is well known that *Mycobacterium leprae* are associated with peripheral nerves. Electron microscopic studies, for example, clearly show large numbers of bacilli within the cytoplasm of Schwann cells ([5]). Schwann cells are involved in the synthesis of the characteristic gangliosides present in peripheral nerves ([9]).

Many nine-banded armadillos develop disseminated (lepromatous) leprosy when inoculated with *M. leprae* ([6]). The ubiquity of gangliosides in animal tissues and their possible involvement in neurofunction led us to investigate ganglioside patterns in *M. leprae*-infected armadillo tissues.

MATERIALS AND METHODS

Experimental animals. Armadillos were inoculated intravenously with suspensions of *M. leprae* and maintained in the animal care facility at the National Hansen's Disease Center, Carville, Louisiana, U.S.A. After signs of dissemination appeared, the animals were sacrificed under anesthesia by cardiac exsanguination. The tissues were removed aseptically and stored at −20° or −80°C before processing. The tissues were examined histologically by acid-fast staining for the presence of *M. leprae*. Tissue from normal (non-inoculated) armadillos served as controls.

Preparation of bacilli. *M. leprae* were separated from the liver tissue of an experimentally infected armadillo, as described previously ([11]).

Extraction of tissue ganglioside. Each tissue sample and the purified bacilli were homogenized in 10 volumes of acetone, centrifuged at 500 × *g* for 10 min, and the residue collected. The residue was extracted successively with 10 volumes each of chloroform-methanol (2:1), chloroform-methanol (1:1), and chloroform-methanol (1:2). The chloroform-methanol extracts were combined and concentrated *in vacuo* at 45–50°C, then taken to dryness under a stream of nitrogen. The dried samples (crude lipid fractions) were further extracted according to the partitioning procedure of Folch, *et al.* ([3]) unless otherwise noted.

Thin-layer chromatographic (TLC) analysis. Aliquots of the ganglioside extracts along with the appropriate standards were applied to silica gel plates (0.25 mm; E. Merck, Darmstadt, Germany) and developed in chloroform-methanol-water (60:35:8). Gangliosides were located by spraying the plates with resorcinol reagent ([13]) and heating them at 110°C for 15 min.

GM3 Ganglioside purification. A crude lipid extract from 100 g *M. leprae*-infected armadillo liver was submitted to mild alkaline hydrolysis by suspending the extract in 10 ml of 0.6 N NaOH in methanol, and incubating at 37°C for 16 hr ([1]). The sample was neutralized with 1 N methanolic HCl, then dried, dialyzed thoroughly against water, and lyophilized. The Folch partition

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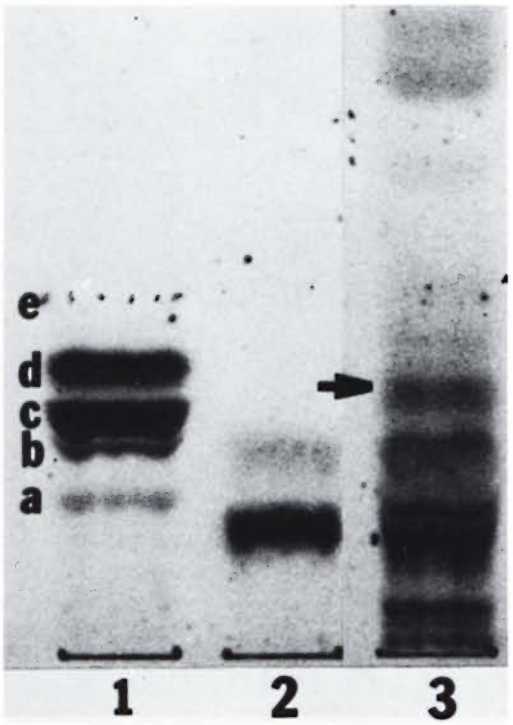

FIG. 1. Thin-layer chromatographic patterns of armadillo-tissue-derived gangliosides in sciatic nerve. (Extraction procedure and solvent system are described in text.) 1 = standard ganglioside mixture (in order of increasing mobility): a = GD1a, b = GM1, c = GM2, d = GM3, e = G7; 2 = noninfected sciatic nerve; 3 = sciatic nerve from lepromatous armadillo; ➧ = GM3 ganglioside in infected tissue extract.

was omitted for this large sample of lipid extract.

Two g of the lyophilized crude lipids from the alkaline hydrolysis was dissolved in 30 ml of chloroform-methanol (2:1), and the suspension was sonicated and centrifuged. The supernatant was retained and the precipitate was washed twice: first with 10 ml of chloroform-methanol (2:1) and then with 5 ml of the same solvent. The clear supernatant fractions were combined (43 ml). Forty ml of this extract was applied to a DEAE-cellulose column (Cellex D; Bio-Rad Corp., Richmond, California, U.S.A.) pretreated according to the procedure of Rouser, *et al.* ([12]).

The Cellex D column was eluted with the following solvents: 500 ml of chloroform-methanol (2:7); 500 ml of the same solvent containing 0.01 M sodium acetate; 500 ml of the same solvent containing 0.02 M sodium acetate. Ten ml fractions were collected. The second and third eluates were combined, evaporated, dialyzed against water, and lyophilized. Final purification of the GM3 ganglioside from the lyophilized product was achieved by gradient elution on a Bio-Sil A column ([10]).

Enzymatic treatment of purified GM3 ganglioside. Ten μg of GM3 ganglioside was incubated with 0.1 unit of neuraminidase (Type VI, *C. perfringens*; Sigma Chemical Co., St. Louis, Missouri, U.S.A.) in 0.2 ml of 0.1 M Na acetate buffer, pH 5.5, overnight at 37°C. After incubation, 1 ml of chloroform-methanol (2:1) was added. The denatured protein formed between the interphase was removed, and the upper and lower layers were combined, evaporated, and analyzed by TLC. The procedure for β-galactosidase treatment of the lactosylceramide has been described elsewhere ([8]).

RESULTS AND DISCUSSION

Thin-layer chromatographic patterns of the gangliosides extracted from normal and lepromatous armadillo tissues are shown in Figures 1 and 2. There seemed to be significant increases of several gangliosides in the infected tissues. However, there was an apparent preferential increase of a ganglioside band which migrated slightly slower than the authentic human GM3 ganglioside isolated from erythrocytes. This ganglioside was isolated by column chromatography, as described in Materials and Methods, and further TLC (Fig. 3) revealed several GM3-like species. A sample of the purified glycolipid was subjected sequentially to enzymatic hydrolysis by neuraminidase and β-galactosidase. A thin-layer separation of the hydrolysis products yielded lactosylceramide and glucosylceramide, indicating that the glycolipid is a GM3 ganglioside (Fig. 4).

The TLC patterns of infected lymph node and sciatic nerve showed not only increased GM3 compared to uninfected control tissues but also a concomitant increase in a ganglioside band similar in mobility to the GM1 or GM2 standard. Several other unidentified, faster-moving bands were also present in the sciatic nerve. In the liver there was an apparent reduction of two ganglioside bands—one similar in mobility to GM2

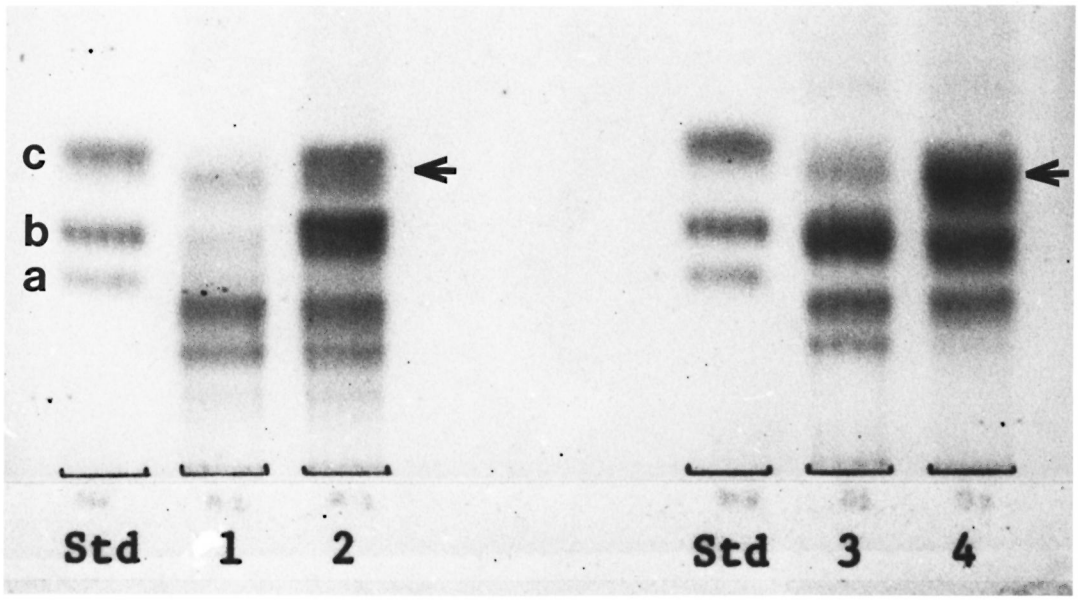

FIG. 2. Thin-layer chromatographic patterns in lymph node and liver. Standard: a = GM1, b = GM2, c = GM3; 1 = lymph node, normal armadillo; 2 = lymph node, lepromatous armadillo; 3 = liver, normal armadillo; 4 = liver, lepromatous armadillo; ➧ = GM3 ganglioside in the affected tissues.

and another band somewhat slower than GM1. Ganglioside concentrations and species distribution normally vary considerably among tissues and this variation might account for a number of the differences observed. The ganglioside patterns may also be influenced by the degree of *M. leprae* infection of the tissues. Acid-fast

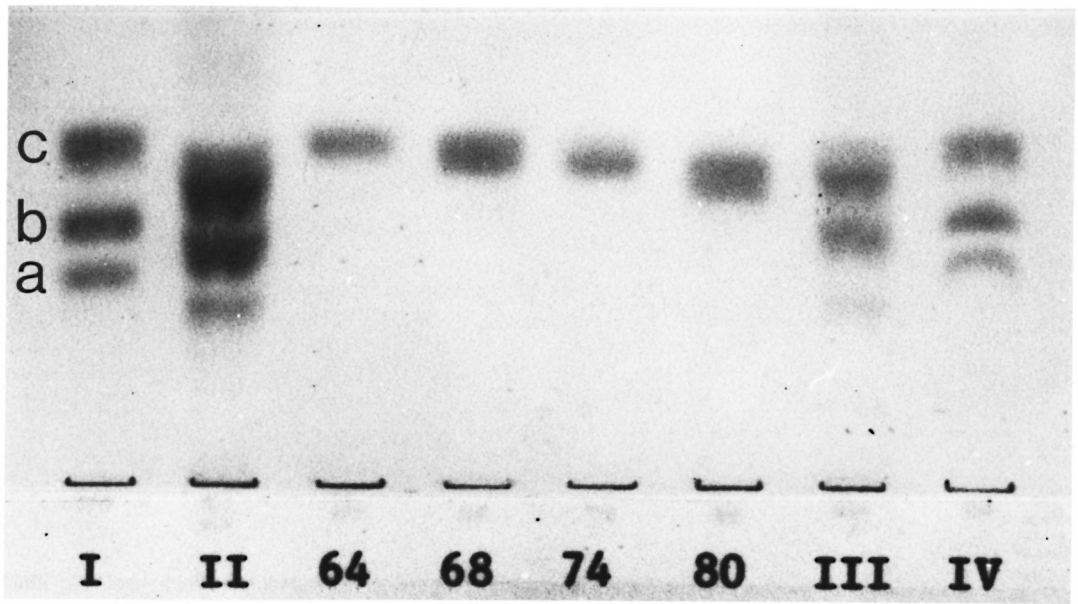

FIG. 3. Thin-layer chromatogram of GM3 ganglioside after column purification (described in text). I = standard mixture: a = GM1, b = GM2, c = GM3; II = before DEAE and Bio-Sil A chromatography; III = after DEAE, before Bio-Sil A; IV = standard mixture. Fractions 64, 68, 74, and 80 represent species of GM3 after Bio-Sil A chromatography as differentiated by their mobility on thin-layer plate.

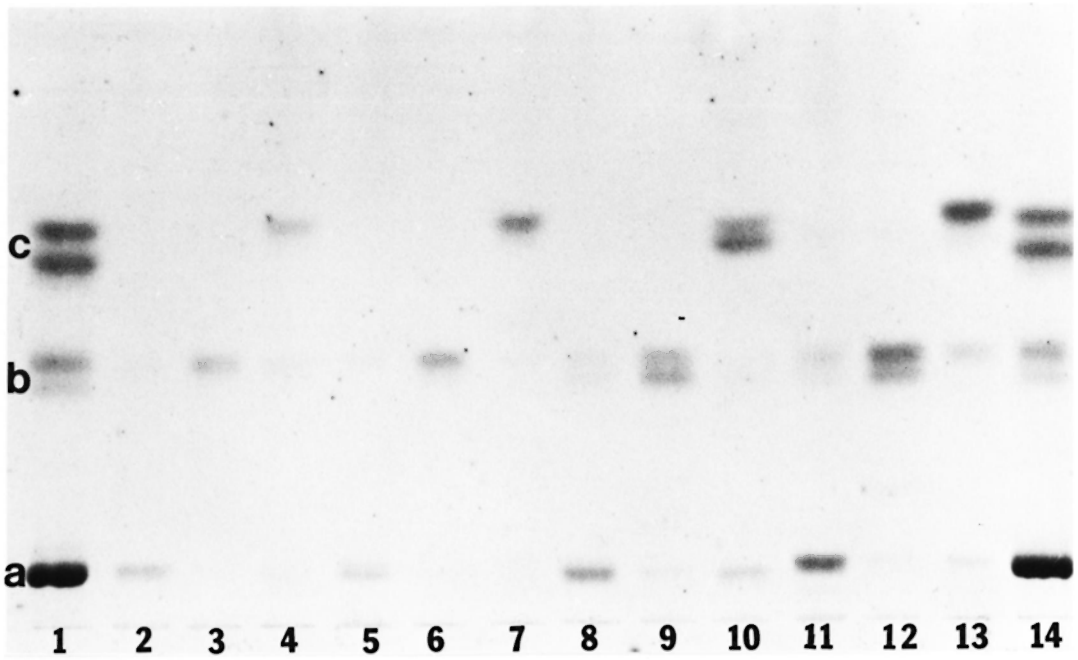

FIG. 4. Thin-layer chromatogram showing identification of GM3 ganglioside by enzymatic hydrolysis of column eluates. a = GM3, b = lactosylceramide, c = glucosylceramide. Columns 1 and 14 are standard ganglioside mixtures. 2 = F68; 3 = F68 + neu; 4 = F68 + neu + β-gal; 5 = F74; 6 = F74 + neu; 7 = F74 + neu + β-gal; 8 = F80; 9 = F80 + neu; 10 = F80 + neu + β-gal; 11 = standard, GM3; 12 = GM3 + neu; 13 = GM3 + neu + β-gal (neu = neuramidinase; β-gal = β-galactosidase; F = fraction number).

stains revealed numerous bacilli in the lymph node and nerve, while the liver contained comparatively fewer organisms.

These findings suggest that host cell metabolism was altered by the leprosy bacilli and, consequently, the constituent glycosphingolipids of the tissues were changed. (GM3 Gangliosides were not detected in lipids extracted in an identical fashion from purified *M. leprae.*) Changes in the ganglioside composition of cells are known to occur as a result of external stimuli. For example, cultured HeLa cells exposed to the short-chain, fatty acid butyrate show an increased biosynthesis of GM3 ganglioside as a result of induced lactosylceramide sialytransferase activity in the cells ([2]).

Several mechanisms could account for the differences in ganglioside patterns between lepromatous and normal armadillo tissues. The specific glycosyltransferases responsible for ganglioside synthesis may be induced by the presence of *M. leprae* in the tissue, thereby resulting in increased ganglioside biosynthesis. Cellular infiltrates into the tissue (macrophages, lymphocytes) may express these enzymes as they interact with *M. leprae,* thereby increasing their ganglioside synthesis. More simply, the inherent ganglioside patterns of the infiltrating cells could be reflected in altered ganglioside patterns of the infected tissues. Finally, the changes observed may merely be secondary to tissue degeneration or inflammation associated with infection with *M. leprae.*

From these limited observations it would be premature to draw any conclusions concerning the significance of altered ganglioside concentrations in lepromatous armadillo tissue. However, their association with neurological dysfunction, involvement as specific, surface membrane receptors, and their antigenic properties provide a reasonable basis for continued interest in these compounds in leprosy.

SUMMARY

Gangliosides derived from tissues of normal and lepromatous armadillos were examined by thin-layer chromatography. The

ganglioside patterns produced by the *Mycobacterium leprae*-infected tissues varied from that of the normal tissue. Although increased levels of several gangliosides were observed in the infected tissues, there was an apparent preferential increase in GM3 gangliosides as determined by column chromatography and enzymatic hydrolysis.

RESUMEN

Usando la cromatografía en capa fina, se examinaron los gangliósidos derivados de los tejidos de armadillos normales y los derivados de armadillos lepromatosos.

El patrón de los gangliósidos producidos por los tejidos infectados con *Mycobacterium leprae* fue diferente del producido por los armadillos normales. Aunque varios gangliósidos derivados de los tejidos infectados mostraron un incremento en su concentración, los gangliósidos GM3 mostraron un incremento aparentemente preferencial, según se determinó por cromatografía en columna e hidrólisis enzimática.

RÉSUMÉ

On a examiné, par une méthode de chromatographie en couches minces, les gangliosides extraits de tissus recueillis chez des tatous normaux et chez des animaux atteints de lèpre. Le profil des gangliosides produits par des tissues infectés par *Mycobacterium leprae* étaient différents de ceux observés dans les tissus normaux. On a observé une augmentation des taux de plusieurs gangliosides dans les tissus infectés. Il est toutefois apparu que cette augmentation semblait porter davantage sur les gangliosides GM3, tels qu'on peut les mesurer par la chromatographie sur colonne et par l'hydrolyse enzymatique.

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