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Membrane Glycolipid and Phospholipid Composition of Lipopolysaccharide-Responsive and -Nonresponsive Murine B Lymphocytes

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Neutral glycolipids, gangliosides, and phospholipids present on membranes of unstimulated or lipopolysaccharide (LPS)-stimulated B cells were analyzed in LPS-responsive C3H/HePAS and LPS-nonresponsive C3H/HeJ mice. In the set of neutral glycolipids, asialo G_{M1} reacted preferentially with galactose oxidase but was not detectable with monospecific antibodies during immunocytofluorescence analysis. Another, more polar, neutral glycolipid appeared exclusively after stimulation of responsive B cells. Among the membrane gangliosides 1 to 5 that were able to react with galactose oxidase on B cells, ganglioside 3 was not detected in the mutant strain, and its absence was counterbalanced by the presence of a larger amount of ganglioside 1. The biosynthesis of total membrane phospholipids and the balance between phosphatidylethanolamine and phosphatidylcholine were significantly different in the two mouse strains examined and were quantitatively and qualitatively modified during the mitogenic response to LPS.

In the presence of lipopolysaccharide (LPS), resting B lymphocytes are activated to undergo blastogenesis. Despite the large amount of data available on this topic, the actual mechanism of the triggering process is still unclear. It has been established by Jakobovits et al. (17) that plasma membrane components regulate the response of lymphocytes to mitogen stimuli and that the inability of C3H/HeJ B cells to respond to LPS is likely to be due to membrane defects.

It is generally postulated (25) that the activation signal of lymphocyte stimulation is triggered restrictively by cell membrane glycoproteins. Glycolipids, however, must also be considered since very similar carbohydrate moieties (5, 43) could be present on both types of glycoconjugates. A number of authors stressed the importance of glycolipids as receptors for lectins (26), viruses (14), toxins (28), interferon (1), and hormones (19) and their involvement in lymphocyte stimulation (41).

Since it is known that LPS is inserted into the lipid layer of biological membranes (31), local alterations in bilayer organization might be expected to result and to lead particularly to modifications of the phospholipid (PL) metabolism. The importance of the modifications of the cellular levels of certain PLs during mitogenic stimulation of lymphocytes has been highlighted by some authors. The turnover of phosphatidylcholine (PC) has been reported to increase immediately after the stimulation with lectins, and transient accumulation of lysophosphatidylcholine was found to increase guanylate cyclase activity (36) and generate cyclic GMP, a nucleotide involved in mitogenesis.

In our investigations on the glycolipid and PL composition of B-cell membranes from LPS-responsive (C3H/HePAS) and -nonresponsive (C3H/HeJ) mouse strains, we have attempted to provide insight into the molecular basis of the mitogenic responsiveness of B cells to LPS.

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MATERIALS AND METHODS

Animals. Mice from the inbred strains C3H/HePAS and C3H/HeJ (age, 7 to 10 weeks) were obtained from the Institut Pasteur, Paris, France. HY/CR hybrid rabbits were from Charles River, France.

Reagents and standards. En^{3}Hance spray was from New England Nuclear Corp., Boston, Mass. LPS was extracted from smooth cells of *Salmonella cholerae suis* (serotypes 6_2, 7, and 14) by the phenol-water procedure (46). PC (type XI-E, from egg yolk), phosphatidylethanolamine (PE; type III, from egg yolk), phosphatidylserine (PS; from bovine brain), lysophosphatidylcholine (type I, from egg yolk), and sphingomyelin (SM; from bovine brain) PL standards, as well as methylated bovine serum albumin, albumin, and cholesterol, were purchased from Sigma Chemical Co., St. Louis, Mo.

The designations of Svennerholm (42) for gangliosides are as follows: G_{M3}, II^3-*N*-acetylneuraminosyllactosylceramide; G_{M2}, II^3-*N*-acetylneuraminosylgangliotriaosylceramide; G_{M1}, II^3-*N*-acetylneuraminosylgangliotetraosylceramide; G_{D3}, II^3-(*N*-acetylneuraminosyl)2-lactosylceramide; G_{D1a}, IV^3-*N*-acetylneuraminosyl, II^3-*N*-acetylneuraminosylgangliotetraosylceramide; G_{D1b}, II^3-(*N*-acetylneuraminosyl)2-gangliotetraosylceramide; G_{T1b}, IV^3-*N*-acetylneuraminosyl-II^3-(*N*-acetylneuraminosyl)2-gangliotetraosylceramide. Gangliosides G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b} (from bovine brain) and G_{D3} (from buttermilk) were purified by the method of Fredman et al. (8). Ganglioside G_{M3} (from human liver) was purified by the method of Seyfried et al. (35). Chemical structure and homogeneity were assessed as described by Ghidoni et al. (10). Purities were over 99%. Ganglioside concentrations were determined by a sialic acid assay (41). Gangliotetraosylceramide (AsG_{M1}) was prepared by hydrolysis of G_{M1} in 0.1 N HCl at 80°C for 1 h and purified by silica gel 100 column chromatography (10).

Isolation of Ig$^+$ cells. Splenic cells were prepared as

described previously (2). Erythrocytes were removed by treatment of the cells (10^8 cells per ml) with 0.83% ammonium chloride for 15 min at 0°C. After two washings (10 min, 400 × *g*), cell pellets were diluted to a density of 5×10^6 cells per ml in RPMI medium supplemented with 20% fetal calf serum. Splenic macrophages were allowed to attach to 100-mm plastic petri dishes (Corning tissue culture dishes) by incubation (5×10^7 splenic cells per plate) at 37°C for 2 h under a 5% CO_2 atmosphere. Immunoglobulin-bearing (Ig^+) cells were isolated from the nonadherent population by the technique of Wysocki and Sato (47). Macrophage-depleted cells (10 ml; 5×10^6 to 1×10^7 cells per ml) were incubated for 70 min at 4°C in polystyrene bacteriological petri dishes previously coated with purified rabbit anti-mouse immunoglobulin G (IgG) antibodies. The nonadherent population was then discarded, and Ig^+ cells were recovered by gentle scraping of the dishes.

Radioactive labeling of cell surface glycoconjugates. Ig^+ cells (5×10^6 cells per ml) from the two mouse strains were cultured for 14 h, either with or without LPS (10 μg/ml), in RPMI 1640 medium supplemented with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) 2 mM L-glutamine, 100 μg of streptomycin per ml, 100 IU of penicillin per ml, and 5×10^{-5} M 2-mercaptoethanol. Cells were washed three times with phosphate-buffered saline (pH 7.4) and labeled by the method of Gahmberg et al. (9). The cells (5×10^7 cells per ml in phosphate-buffered saline pH 7.0) were incubated for 3 h at 37°C with galactose oxidase (type V from Sigma; 5 IU/ml) under gentle stirring. After two washings in phosphate-buffered saline, sodium [^{3}H]borohydride (1 mCi, 25 Ci/mmol) was added, and the cell suspension (10^8 cells in 1 ml of phosphate-buffered saline; pH 7.4) was stirred for 30 min at room temperature. Cells were washed five times, and incorporated ^{3}H (1.5×10^6 cpm per 10^8 cells) was measured in a liquid scintillation counter.

Production of an anti-AsG_{M1} serum. A rabbit anti-AsG_{M1} serum was prepared by using liposomes containing highly purified AsG_{M1} by the method described by Coulon-Morelec and Buc-Caron (3). Briefly, a liposome suspension (1 ml) containing PC, cholesterol, methylated bovine serum albumin, and AsG_{M1} in a molar ratio of 1:5.5:0.0012:0.13, respectively, was injected intravenously into rabbits three times a week for 4 to 7 weeks. The rabbits were bled (cardiac puncture) 5 days after the 15th injection. The activity and the specificity of the anti-AsG_{M1} serum were determined by semiquantitative methods of passive agglutination and complement fixation as previously described (4) and by inhibition of the lysis of liposomes containing AsG_{M1} (37).

TLC immunostaining. Purified neutral glycolipids (NGL) were chromatographed on aluminium-backed high-performance thin-layer chromatography HPTLC plates (E. Merck AG, Darmstadt, Federal Republic of Germany). The plates were overlaid with rabbit anti-AsG_{M1}, followed by peroxidase-linked sheep anti-rabbit immunoglobulin (20). After several washings, the plates were exposed to the enzyme substrate 4-chloro-1-naphthol.

Separation of neutral and acidic lipids. ^{3}H-labeled cells (3×10^7 to 1×10^8) were extracted successively for 5 min with 20 volumes of chloroform-methanol (2:1 [vol/vol]) and chloroform-methanol-water (1:2:0.15 [vol/vol]) at 50 and 37°C. Total lipids (4.6×10^5 cpm per 10^8 cells) were recovered after evaporation of the combined extracts under a nitrogen stream. Nonganglioside lipids (fraction F1) were separated from gangliosides (fraction F2) after silicic acid chromatography (Unisil 100/200 mesh; Clarkson Chemical Co., Williamsport, Pa.) (16) followed by ion-exchange chromatography on DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) (23).

Purification and analysis of NGL. NGL present in fraction F1 were dried over P_2O_5, peracetylated with 500 μl of pyridine-acetic anhydride (3:2 [vol/vol]) at 20°C for 18 h, and purified by chromatography on Florisil (magnesia-silica gel, 50/100 mesh; Fisher Scientific Co., Pittsburgh, Pa.) (32). Three fractions, corresponding to cholesterol, diglycerides, and PLs, were removed. The radioactive labeling of these non-galactosamine-containing lipids is due to a nonspecific hydrogenation (40). The eluted glycolipid fraction was deacetylated with 0.2 N methanolic sodium hydroxide (750 μl) at 37°C for 4 h. The reaction mixture was then neutralized with Dowex 50 W-X8 (H^+) cation-exchange resin, and NGL were chromatographed on a Sephadex LH-20 column to remove nonlipid contaminants (24). Purified [^{3}H]NGL (1.4×10^5 cpm/10^8 labeled cells) were eluted from the column with chloroform-methanol (2:1 [vol/vol]) and dried under nitrogen.

Individual NGL were analyzed by one-dimensional HPTLC on Kiesel-gel 60 plates (20 by 10 cm) developed with chloroform-methanol-water (60:30:5 [vol/vol]). ^{3}H-labeled NGL samples (10^4 cpm) or reference mixtures (10 nmol of individual glycolipids) were applied to the plates. Radioactive bands were visualized by autoradiography (Kodak X-ray film, exposure for 5 days at −70°C) after spraying the plates with En^{3}Hance spray. NGL references were visualized with alpha-naphthol.

Purification and analysis of gangliosides. Fraction F2, containing the acidic lipids eluted from the DEAE-Sephadex column with 0.5 M sodium acetate, was desalted on a Sep-Pak C_{18} reverse-phase cartridge (Waters Associates, Inc., Milford, Mass.) by the method of Ledeen and Yu (22). Nonlipid contaminants were removed by chromatography on Sephadex LH-20, as described above for the NGL purification process, and purified ^{3}H-gangliosides (10^4 cpm per 10^8 labeled cells) were isolated.

Analysis of gangliosides was performed on Kiesel-gel 60 plates (10 by 10 cm) either by one-dimensional chromatography with solvent I (chloroform–methanol–0.2% aqueous $CaCl_2$ [55:45:10, vol/vol]) or solvent II (chloroform–methanol–2.5 M NH_4OH [60:40:9, vol/vol]) or by two-dimensional chromatography with a first development in chloroform–methanol–0.2% aqueous $CaCl_2$ (50:40:10 [vol/vol]) and a second development, in a perpendicular direction, with *n*-propanol–17 M NH_4OH–water (6:2:1 [vol/vol]). ^{3}H-ganglioside samples (5,000 cpm) or unlabeled references (2 to 3 nmol) were applied to the plates. Radioactive spots were detected as described above for NGL analysis. Ganglioside references were visualized with resorcinol (21). Relative radioactivities of individual gangliosides were estimated by scanning the radioautogram with a scanning densitometer (model PHI 6; Vernon) or by measuring in a liquid scintillation counter the radioactivity of the silica gel samples scraped out from the areas corresponding to the spots, and the gangliosides were then suspended in tetrahydrofuran (0.2 ml).

PL labeling, extraction, and analysis. Erythrocyte-depleted splenic cells (10^7 cells per ml; 7 ml) from the two mouse strains were incubated for 1 h at 37°C in RPMI 1640 containing $^{32}P_i$ (40 μCi/ml). RPMI medium (7 ml) either with or without LPS (20 μg/ml) was added, and the cells were cultured at 37°C for an additional 1 to 19 h. Cells were washed twice with cold (0°C) balanced salt solution (8 ml), and the Ig^+ cell population was prepared as described above. Total lipids (4.1×10^4 cpm), extracted from 10^6 ^{32}P-labeled

cells as described above, were dissolved in 0.6 ml of chloroform-methanol (2:1 [vol/vol]) and partitioned by adding 1/5 volume of distilled water, by the method of Folch (6). After evaporation of the lower phase to dryness, labeled PLs (1.6×10^4 cpm) were analyzed on Kiesel-gel 60 plates (10 by 10 cm) either by one-dimensional chromatography (two ascending runs) with acetone-petroleum ether (1:3 [vol/vol]) and chloroform-methanol-acetic acid-water (50:25:7:3 [vol/vol]) successively or by two-dimensional chromatography with chloroform–methanol–25% ammonia (65:25:5 [vol/vol]) and chloroform-methanol–acetone-acetic acid-water (30:10:40:10:5 [vol/vol]). [^{32}P]PLs (5,000 cpm) and reference mixtures (20 nmol of individual PLs) were applied to the plates. Radioactive spots were detected by autoradiography (Kodak RP Royal X-Omat X-ray film, exposure for 7 days). References were visualized after heating (190°C, 30 min) the plates sprayed with 20% ammonium sulfate. The distribution of radioactivity in individual PLs was determined with a scanning densitometer as described above.

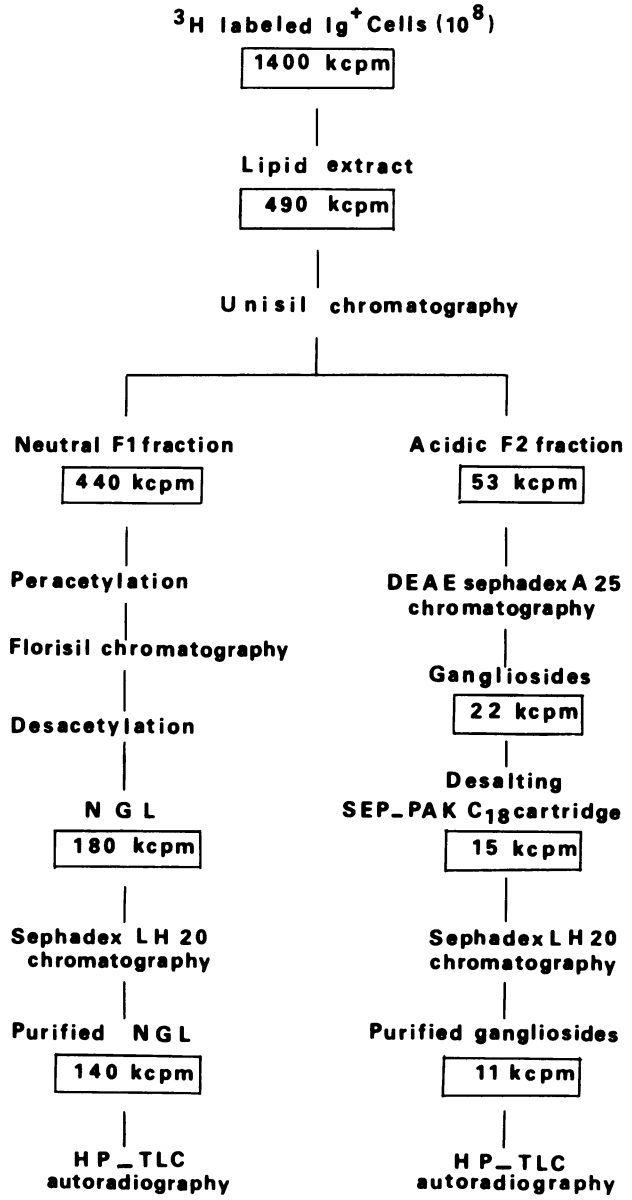


FIG. 1. Distribution of ^{3}H radioactivity during the procedures of purification of neutral glycolipids and gangliosides of Ig$^+$ cells from unstimulated C3H/HePAS mice.

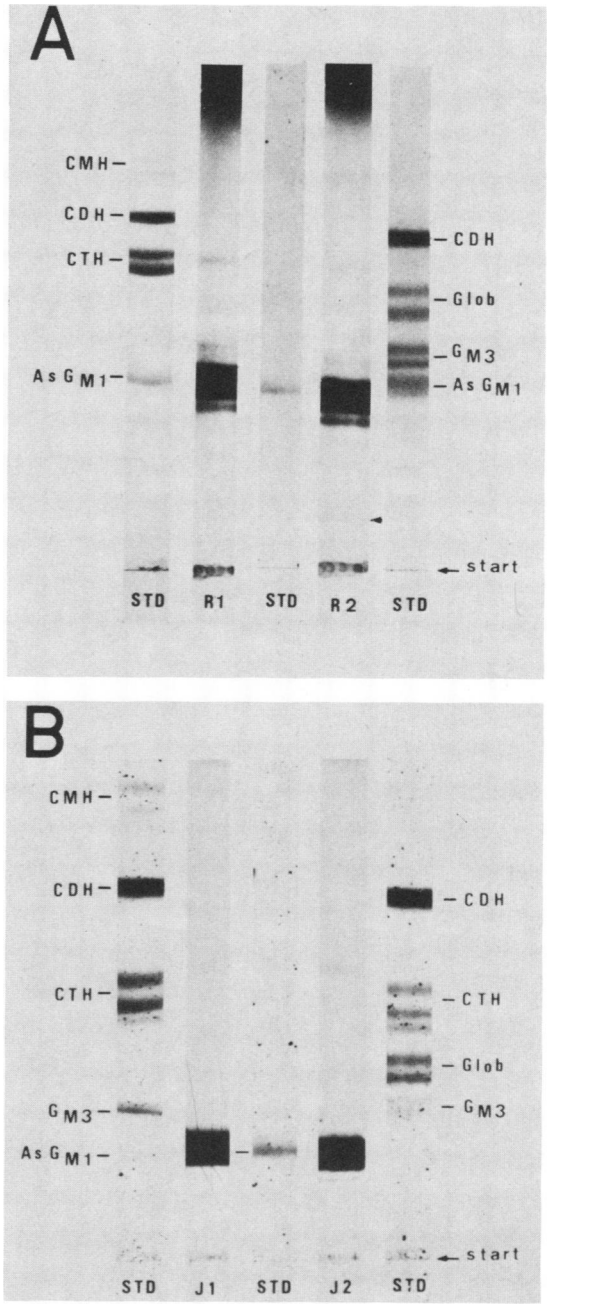


FIG. 2. Autoradiograms of radiolabeled neutral glycolipids separated by HPTLC. ^{3}H-labeled NGL (10^4 cpm) extracted from resting (lanes R1 and J1) and LPS-treated (lanes R2 and J2) Ig$^+$ splenocytes from C3H/HePAS (A) and C3H/HeJ (B) mice were applied to Kiesel-gel 60 plates and chromatographed with chloroform-methanol-water (60:30:5 [vol/vol]) NGL references (lanes STD) were stained with alpha-naphthol reagent.

RESULTS

To analyze the effect of mitogenic stimulation on glycolipid patterns, immunoglobulin-bearing lymphocytes (Ig$^+$ cells) isolated from spleen cells of C3H/HePAS (LPS-

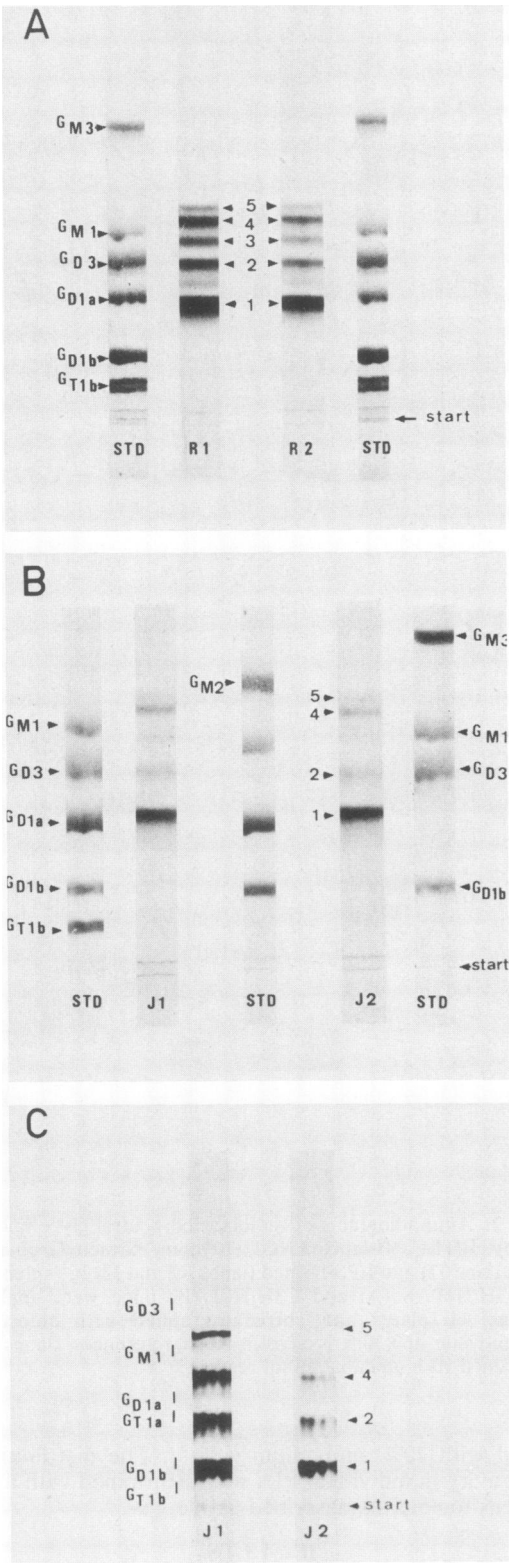


FIG. 3. One-dimensional HPTLC of radiolabeled gangliosides. ^{3}H-labeled gangliosides (5,000 cpm) extracted from resting (lanes R1 and J1) and LPS-treated (lanes R2 and J2) Ig^+ splenocytes from C3H/HePAS (A) and C3H/HeJ (B and C) mice were applied to Kiesel-gel 60 plates. Chromatograms were developed with solvent I (A and B) or with solvent II (C), as described in Materials and Methods. Radioactive bands were visualized by autoradiography. Ganglioside references (lanes STD) were stained with resorcinol reagent.

responsive) and C3H/HeJ (LPS-nonresponsive) mice were incubated for 14 h either with or without the addition of LPS in the culture medium. External labeling of galactose and *N*-acetylgalactosamine constituents of membrane glycolipids was then carried out. The efficiencies of labeling of 10^8 Ig^+ cells from C3H/HePAS incubated with LPS (1.3×10^6 cpm) or without LPS (1.4×10^6 cpm) and from C3H/HeJ incubated with LPS (1.6×10^6 cpm) or without LPS (1.6×10^6 cpm) were very similar. Labeled NGL and gangliosides were extracted, separated on ion exchangers (Fig. 1), and analyzed by HPTLC followed by autoradiography. Migration distances were compared with those of standards visualized with specific reagents.

Neutral glycolipids. Significant differences in the amount of radioactivity incorporated into neutral glycosphingolipids were observed between resting B lymphocytes from C3H/HePAS and C3H/HeJ mice (1.4×10^5 and 0.75×10^5 cpm/10^8 Ig^+ cells, respectively). These two cell populations exhibited similar patterns of labeled NGL (Fig. 2, lanes R1 and J1). The position of the most prominent band was identical to that of a gangliotetraosylceramide (asialo G_{M1}) standard. The unambiguous identification of this compound to AsG_{M1} was further confirmed by the reactivity of the corresponding band of the chromatogram with anti-AsG_{M1} antibodies in an immunostaining assay. It should be mentioned, however, that although AsG_{M1} was the main glycolipid component accessible to galactose oxidase, we were unable to visualize it on Ig^+ cells from the two mouse strains after successive incubations of the cells with specific anti-AsG_{M1} and fluoresceinated anti-rabbit IgG sera, by the procedure described in Materials and Methods.

In addition to AsG_{M1}, three other, much more weakly labeled, NGL were present on the cells from the two mouse strains (Fig. 2) and were detected by HPTLC in the areas of globotriaosylceramide, globotetraosylceramide, and G_{M3} ganglioside. When cultures were carried out in the presence of LPS, neither qualitative nor quantitative changes were observed in the labeling pattern of NGL isolated from C3H/HeJ mice (Fig. 2, cf. lanes J2 and J1), whereas an additional minor NGL, migrating more slowly than AsG_{M1} (Fig. 2, arrow on lane R2), appeared on Ig^+ cells from C3H/HePAS mice. Moreover, with the latter strain exclusively, a significant (27%) decrease of the radioactivity incorporated into NGL (the AsG_{M1} area of the chromatograms) was also observed during this mitogenic stimulation of the cells (2,233 versus 1,630 cpm with C3H/HePAS cells; 1,243 versus 1,294 cpm with C3H/HeJ cells).

Acidic glycolipids (gangliosides). The average radioactivity incorporated into gangliosides was 12-fold lower (1.1×10^4 versus 1.4×10^5 cpm per 10^8 Ig^+ cells) than that of NGL. Upon one-dimensional HPTLC analysis, the pattern of labeled gangliosides (compounds 1 to 5) from C3H/HePAS cells (Fig. 3A) appeared qualitatively and quantitatively different from that obtained from the C3H/HeJ strain (Fig. 3B). The migration rates (in solvent I) of the three major gangliosides (1, 2, and 4) common to both mouse strains,

relative to a G_{M3} ganglioside reference from human spleen, were 0.4, 0.55, and 0.7, respectively (Fig. 3A and B). Comparison of the migration of the gangliosides from the mutant mouse strain in neutral and alkaline solvent systems (Fig. 3B and C) revealed that these gangliosides behaved differently from those of bovine brain. Indeed, in solvent II (Fig. 3C), ganglioside 1 migrated in the area of G_{T1b} brain ganglioside, whereas its migration in solvent I (Fig. 3B) was similar to that of G_{D1a}. Comparable differences between the two solvent systems were also observed with the two other major gangliosides, 2 and 4.

Comparisons of the ganglioside patterns of the various cell populations were made easier by the use of two-dimensional HPTLC. The major qualitative difference between resting cells from the two mouse strains concerned ganglioside 3.

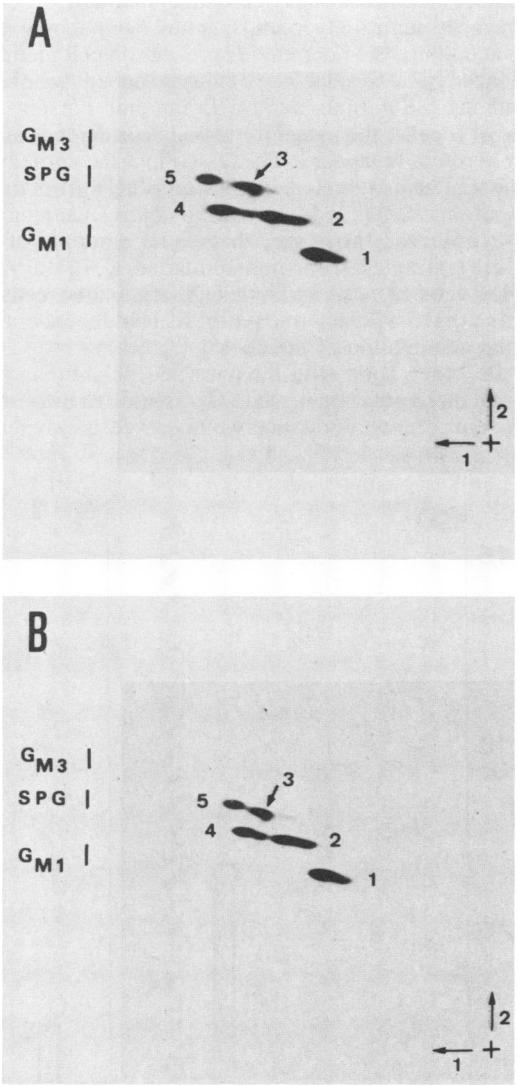


FIG. 4. Two-dimensional HPTLC of C3H/HePAS gangliosides. ^{3}H-labeled gangliosides from resting (A) and LPS-treated (B) Ig^+ splenocytes, were applied to Kiesel-gel 60 plates, which were developed first (direction 1) with chloroform–methanol–0.2% aqueous $CaCl_2$ (50:40:10 [vol/vol]), and afterwards (direction 2) with *n*:propanol–17 M NH_4OH–water (6:2:1 [vol/vol]). Migrations of ganglioside standard in the second solvent system are indicated. Radioactive spots were visualized by autoradiography.

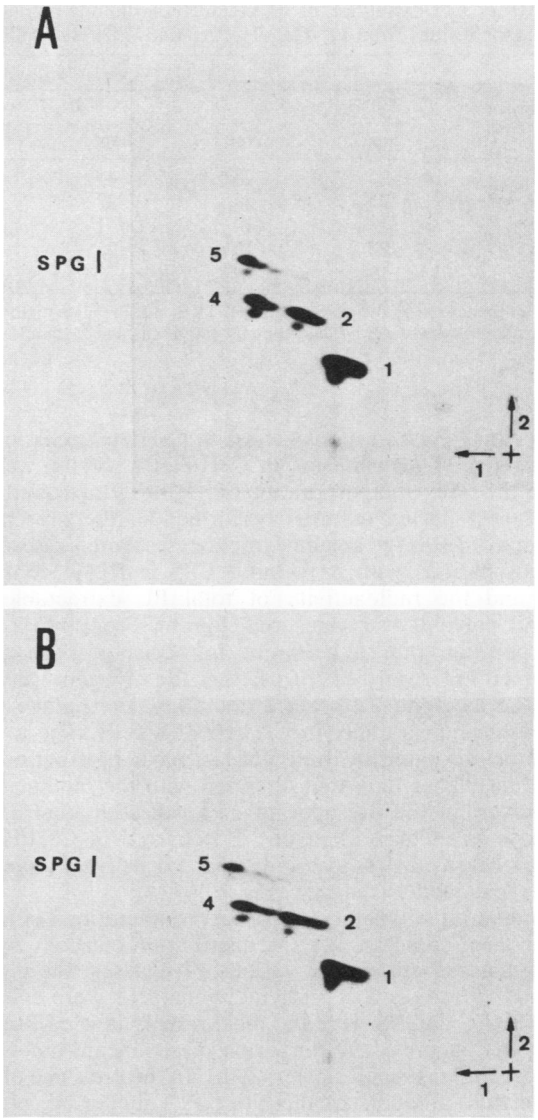


FIG. 5. Two-dimensional HPTLC of C3H/HeJ gangliosides. ^{3}H-labeled gangliosides from resting (A) and LPS-treated (B) splenocytes from the LPS-unresponsive (C3H/HeJ) mouse strain were analyzed as described in the legend to Fig. 4.

This compound, present on C3H/HePAS cells (Fig. 4A, arrow), was not detected in the gangliosides isolated from C3H/HeJ cells (Fig. 5A). Less prominent but significant differences were also observed in the relative amounts of the various ganglioside constituents of the two strains. The percentage of ganglioside 1 was higher in C3H/HeJ (43 to 45%) than in C3H/HePAS (25 to 28%) (Table 1). The lower amount of compound 1 in the latter strain was counterbalanced by the presence of compound 3 (14 to 18%) which was almost absent (1 to 3%), as mentioned above, from the Ig^+ cells of the mutant strain.

As regards the influence of LPS stimulation, no significant change was observed in the ganglioside pattern of Ig^+ cells from the two mouse strains when cultured for 14 h in the presence of the mitogen (Fig. 4B and 5B).

Incorporation of ^{32}P into PL molecules. To determine whether modifications of the PL metabolism could be corre-

TABLE 1. Distribution of ^3H radioactivity among individual gangliosides from Ig^+ C3H/HePAS and C3H/HeJ cells

Ganglioside	Relative ^3H-ganglioside radioactivity (%) in[a]:			
	C3H/HePAS cells		C3H/HeJ cells	
	Expt 1	Expt 2	Expt 3	Expt 4
1	25	28	45	43
2	25	29	22	25
3	18	14	1	3
4	23	19	21	21
5	9	10	11	8

[a] After separation by two-dimensional HPTLC, the ^3H radioactivity of each ganglioside was measured by densitometry (Materials and Methods).

lated with LPS unresponsiveness in C3H/HeJ cells, or with LPS-triggered mechanisms in C3H/HePAS cells, we compared the rates of incorporation of ^{32}P into PLs from the two cell types, during cultures performed in the presence or absence of LPS. Ig^+ splenic lymphocytes were incubated for various periods with or without LPS in RPMI containing $^{32}P_i$, and the radioactivity of total PL extractable with organic solvents was measured (Fig. 6). From 0 to 2 h, the incorporation rate of ^{32}P into Ig^+ cells of both origins remained low regardless of whether the mitogen was added into the medium. Between 2 and 19 h, the uptake of ^{32}P increased more rapidly in C3H/HePAS cells. The radioactivity incorporated in these cells, after a 19-h period, was threefold higher than that obtained with the mutant strain. Moreover, in the presence of LPS, an additional 2.4-fold increase in incorporation of ^{32}P occurred in C3H/HePAS cells, whereas the radioactivity of the cells from the mutant strain remained unchanged (Fig. 6).

To determine whether the observed alterations of the PL metabolism could modify the distribution of newly synthesized material among the various PL classes, the percentages of ^{32}P radioactivity into individual PLs were examined (Fig. 7). PC and PS were the most rapidly labeled PLs (1 h) (Fig. 7A), whereas the formation of significant amounts of SM and PE was much slower (19 h). In the presence of LPS, the relative amounts of the latter PLs increased, whereas those of PC and PS decreased. Analysis of individual phospholipids from resting C3H/HeJ cells (Fig. 7B) indicated that the amounts of PC and SM were lower and higher, respectively, than those found in resting cells from C3H/HePAS mice and comparable to those found in LPS-stimulated cells of the latter strain. The relative amounts of individual PLs in C3H/HeJ cells was not significantly modified after incubation of the cells with LPS.

DISCUSSION

At the present time, little is known about the ganglioside composition of resting murine B lymphocytes. Although experiments involving labeling of gangliosides from C3H/Tif *nu/nu* splenocytes, fractionated CBA/J splenic B cells, or B-cell hybridoma have been carried out (30), the data presented were obtained after stimulation and metabolic labeling of the cells and cannot therefore be extended to resting B lymphocytes. On the other hand, labeling of cell surface constituents by enzymatic procedures can be applied to resting cells and allows, moreover, the restriction of the analysis of glycolipids to those accessible to external agents such as the B-cell mitogen LPS.

In this study, cell surface gangliosides accessible to and able to react with galactose oxidase were labeled with tritium. It is established (9) that only oligosaccharide chains with at least three sugar residues and containing galactose, *N*-acetylgalactosamine, or *N*-acetylglucosamine are susceptible to this cell-surface labeling technique. Extracts from LPS- responsive C3H/HePAS were compared with those from LPS-nonresponsive C3H/HeJ B cells, and extracts from resting and LPS-stimulated C3H/HePAS B cells were also compared.

Five distinct gangliosides (designated 1 to 5 according to their relative chromatographic mobilities) were labeled on Ig^+ cells from LPS-responsive mice. The considerable difference in the chromatographic mobilities of these gangliosides in neutral and alkaline conditions, as compared with those of standard gangliosides from beef brain, suggests the presence of one or more *N*-glycolylneuraminic acid residues in the molecule. The migratory properties of one of these compounds, ganglioside 5, were identical to those of a 2,3-sialosyl-lactoneotetraosylceramide standard.

As regards neutral glycolipids, it has been shown (9) that mono- and dihexoside ceramides present on cell membranes are not accessible to galactose oxidase. Indeed, we observed that, among the various neutral glycolipids present on the surface of B cells, the gangliotetraoside ceramide asialo G_{M1} was preferentially labeled. This glycolipid has already been identified, after metabolic labeling, on B cells from different mouse strains stimulated with various mitogenic agents (11, 30). We observed, however, that equal amounts of asialo G_{M1} were extracted from nonstimulated C3H/HePAS and C3H/HeJ cells as well as from LPS-stimulated cells, thus indicating that asialo G_{M1} biosynthesis is independent of the mitogenic stimulation of the cells.

On the other hand, the biosynthesis of another neutral glycolipid more polar than asialo G_{M1} seems to be correlated to mitogenic stimulation since we observed its presence on

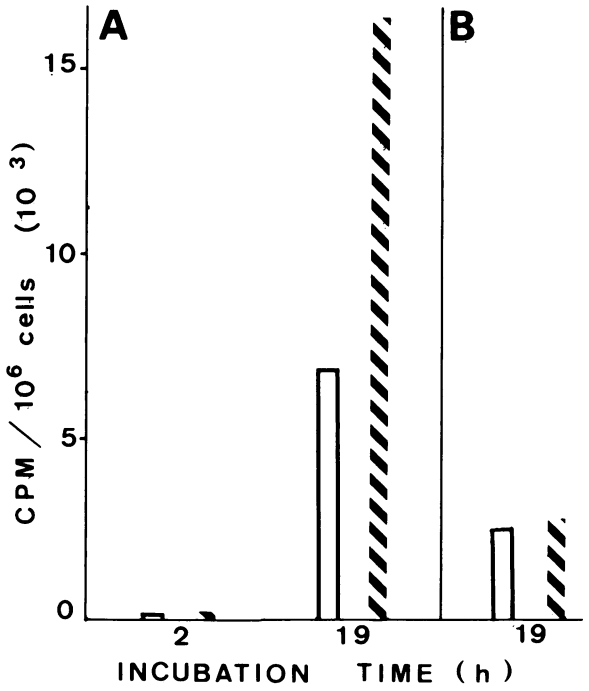
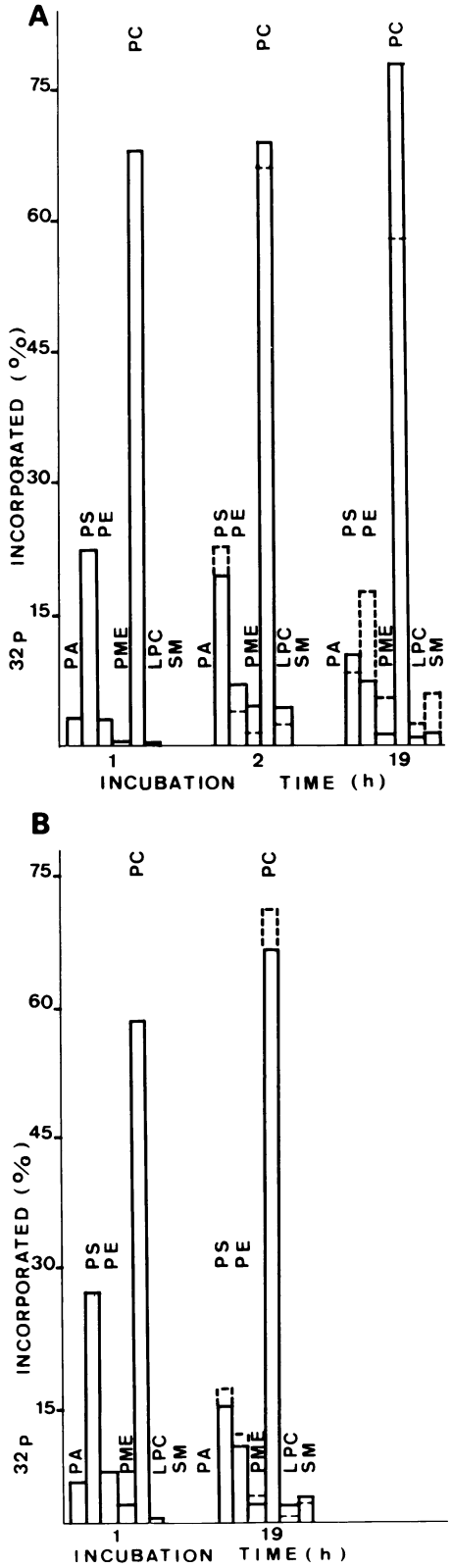


FIG. 6. Incorporation of ^{32}P into PLs. After 2 or 19 h of incubation at 37°C of C3H/HePAS (A) and C3H/HeJ (B) Ig^+ splenocytes with $^{32}P_i$ and with (▨) or without (□) LPS, the radioactivity present in total PL extracts was determined.



LPS-stimulated C3H/HePAS B cells but were unable to detect it on C3H/HeJ cells incubated with the same mitogen, nor on unstimulated B cells from the two mouse strains. It is unknown whether this is specific to the LPS action or could be observed with other B mitogens.

Although exposed on the surface of B lymphocytes from the two mouse strains, we were unable to detect asialo G_{M1} by immunofluorescence, using monospecific anti-asialo G_{M1} antibodies. The inaccessibility of this glycolipid to antibodies on intact or pronase-treated B cells from C3H/HeJ mice has already been reported by Stein et al. (39). Although asialo G_{M1} is present on almost all lymphoid cells, the variation of its accessibility to immunocytochemical reagents led some authors to consider this glycolipid either as a differentiation antigen associated with the maturation of murine natural killer cells (18, 33, 49) or as an oncofoetal antigen, present on T cells from embryonic mice, and progressively decreasing during the appearance of Thy1, Lyt1, and Lyt2 (12, 27). Differences in reactivities between enzymes and anti-glycolipid antibodies have been observed for the first time by Hakomori (13) with a globoside from human erythrocytes, and Young et al. (48) reported recently that reactivities of ^{125}I-labeled anti-asialo G_{M2} with lymphoma subclones were not reflected in surface exposure of asialo G_{M2} as detected by the galactose oxidase procedure. Such a difference between the reactivities of antibodies and galactose oxidase with glycolipids cannot be exclusively due to the differences in the molecular weights of these reagents (160,000 and 76,000, respectively). The affinities of these compounds and the conformation of their binding sites have also to be considered.

Whereas five distinct gangliosides were labeled on Ig^+ cells from LPS-responsive mice, only four of these gangliosides were detected on the corresponding cells obtained from the LPS- nonresponsive mouse strain. Ganglioside 3 could either be absent or occupy a cryptic position inaccessible to the action of galactose oxidase on the surface of cells from the LPS-nonresponsive mouse strain. Furthermore, the percentage of compound 1, the most polar of the five gangliosides, was higher on B cells from the nonresponsive strain, where it accounted for almost half of the ganglioside set. The excess amount of ganglioside 1 present on C3H/HeJ cells was comparable to the amount of ganglioside 3 detected on C3H/HePAS cells. This could be due to the accumulation of compound 1 (the most complex of the gangliosides) on C3H/HeJ B cells as the consequence of a defect in the enzymatic system involved in the metabolic equilibrium between gangliosides 1 and 3.

Results of several studies have suggested a possible role of gangliosides in the mitogenic stimulation of lymphocytes. Sela et al. (34) and Spiegel et al. (38) demonstrated that cross-linking of membrane gangliosides with multivalent ligands results in the coalescence of ganglioside clusters in patches and caps, the formation of which appear to deliver the signal for mitogenic stimulation. The differences in the ganglioside compositions of Ig^+ cells from C3H/HePAS and

FIG. 7. Distribution of ^{32}P in individual membrane PLs. C3H/HePAS (A) and C3H/HeJ (B) Ig^+ splenocytes were incubated with $^{32}P_i$ for 1, 2, or 19 h at 37°C, with (⬚) or without (□) LPS. PL were extracted and submitted to HPTLC analysis as described in Materials and Methods. The distribution (percentage) of ^{32}P radioactivity in individual PLs was determined by scanning the autoradiograms with a scanning densitometer. PA, Phosphatidic acid; LPC, lysophosphatidylcholine; PME, phosphatidylmethylethanolamine.

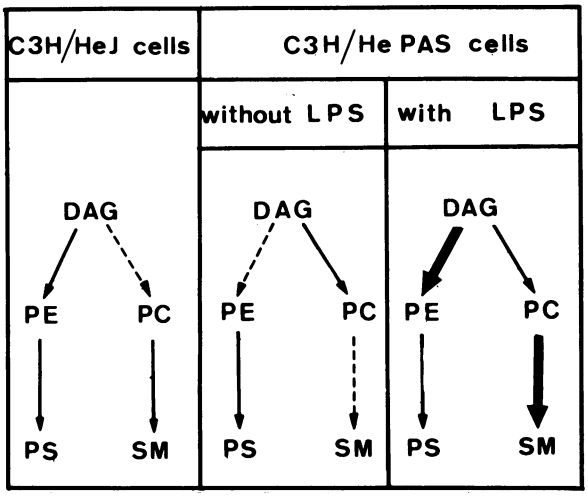


FIG. 8. Comparative scheme of the metabolic pathways of various membrane PLs in B cells from C3H/HeJ and C3H/HePAS mice. DAG, Diacylglycerol; other abbreviations are defined in the legend to Fig. 7 and in the text. Arrows represent slow (----➤), medium (——➤), and fast (➡) metabolic pathways.

C3H/HeJ mice observed in the present study are likely to reflect a difference in the organization of their membranes and may therefore explain the differences in the responsiveness of these cells to LPS.

Chaby et al. (2) have shown that the labeling of C3H/HePAS B cells with a rhodamine-LPS conjugate is significantly higher than that of B cells obtained from the LPS-nonresponsive mouse strain, and they have suggested that this difference could be due to the binding of the LPS to membrane components relevant for the triggering of a mitogenic response. Ganglioside 3, which, according to our present results, is accessible to galactose oxidase exclusively on LPS-responsive cells, belongs to a series of compounds often mentioned as being involved in interactions with external bioactive agents, and it could be the specific LPS-binding site postulated previously. The antigenic specificity of ganglioside 3 should then be similar to that of the putative LPS receptor serologically defined by Forni and Coutinho (7). The preferential binding of LPS to particular membrane gangliosides could reduce the barrier of potential energy of the PL bilayer and allow the biologically active moiety (lipid A) of the endotoxin to insert into the cell membrane. It might be expected that the LPS-ganglioside interaction and the resulting cell stimulation should be critically dependent upon the hydrophobicity and fatty acid composition of lipid A. This assumption is corroborated by observations of marked differences in mitogenic activities of some LPSs of unusual fatty acid composition (29, 45).

As regards the PLs, it is known (44) that a balance between the biosynthesis of PE and PC from diacylglycerol takes place first, followed by the conversion of these PLs into PS and SM, respectively. In the present study, we observed that, after 19 h of culture, the biosynthesis of membrane PLs in resting B cells from C3H/HePAS mice was threefold higher than that in C3H/HeJ cells. Furthermore, the balance between PC and PE was modified in favor of the latter in the mutant mouse strain (Fig. 8). Incubation of cells with LPS was without effect on the mutant, LPS-nonresponsive, C3H/HeJ strain, whereas it induced a marked enhancement (2.4-fold) of PL biosynthesis in LPS-responsive C3H/HePAS mice. Moreover, the presence of LPS favored the formation of PE and SM and disfavored that of PC on B cells from the latter mouse strain (Fig. 8). Although various studies indicate that the cellular levels of some PLs are involved in the mitogenic stimulation of lymphocytes (15, 36), the precise action of these PLs during this process is still unclear and needs further investigation.

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