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# Glycosphingolipids of Purified Human Lymphocytes†

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ABSTRACT: Biochemical analysis of the glycosphingolipids (GSLs) of human lymphocytes revealed qualitative and quantitative variations among purified lymphocytes from different tissues. The major neutral GSLs of tonsil lymphocytes are glucosyl ceramide (CMH), lactosyl ceramide (CDH), trihexosyl ceramide (CTH), and globoside. Thymocytes and peripheral blood lymphocytes (PBL) contain only traces of CTH and globoside, and PBL contain more CMH and CDH per cell than tonsil lymphocytes. Thymocytes and PBL contain relatively large amounts of more complex neutral GSLs that are present in only trace amounts in tonsil lymphocytes. Peripheral blood lymphocytes contained three and five times more lipid-bound sialic acid than thymocytes and tonsil lymphocytes, respectively. Thymocytes and PBL contained mostly hematoside, whereas tonsil lymphocytes contained more complex gangliosides in addition to hematoside. The observed differences in GSL content among these cells may be related to their content of B cells, which comprise approximately 50% of tonsil lymphocytes, 10% of PBL and 0–2% of thymus cells, and/or the known differences in functional capacities of cells in different lymphoid organs. These findings suggest that cell surface GSLs may serve as markers for identification of functional subpopulations of human lymphocytes.

One of the most important advances in immunology in recent years was the recognition of the functional specialization of lymphocyte subpopulations. The use of alloantisera against theta antigen (Reif and Allen, 1966) and the Ly antigens (Cantor and Boyse, 1975) led to the identification of T cells and T cells subsets in the mouse. More recently Huber et al. (1977) and Ahmed et al. (1977) described cell surface antigens present on murine B cell subpopulations. Comparable markers for human lymphocytes are not as well defined but there is evidence for subsets of human T cells (Moretta et al., 1975; Evans et al., 1977).

Our studies of murine lymphocytes (Stein-Douglas et al., 1976; Stein et al., 1977) demonstrated that purified antibodies to glycosphingolipids (GSLs)[1] can be used to identify subpopulations of T cells and immunoglobulin-bearing lymphocytes (B cells). We have undertaken a chemical and immunological study of human lymphocyte GSLs, and we report here differences in the GSL content of normal lymphocytes from different tissues.

## Materials and Methods

### *A. Cell Preparation and Characterization*

*Peripheral Blood Lymphocytes (PBL).* Leukocyte-rich plasma (acid-citrate-dextrose anticoagulant) was obtained by passage of multiple units of human blood from a single donor through a Haemonetics Model 30 cell separator (Haemonetics Corp., Natick, Mass.; Segel et al., 1977). The leukocyte-rich plasma was centrifuged at 150*g* for 8 min at 4 °C to remove platelets and the lymphocyte-rich pellet was mixed with an equal volume of freshly drawn plasma (of the donor blood type) to enhance coagulation. The cell suspension was recalcified by the addition of 0.1 volume of 10% $CaCl_2$ and defibrinated by manual rotation for 40 min at room temperature in an Erlenmeyer flask containing 5 glass beads (2-mm diameter) per 10 mL of cell suspension. The fibrin clot was removed and the cells were diluted with Eagle's minimum essential medium (MEM) to a concentration of $2 \times 10^8$ leukocytes per 25 mL. The diluted cell suspension was centrifuged in aliquots of 25 mL of cells on 10 mL of Ficoll-Hypaque (F-H)

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[1] Abbreviations used: F-H, Ficoll-Hypaque; PBS, phosphate-buffered saline, 0.02 M $PO_4$, pH 7.3, 0.15 M NaCl; FCS, fetal calf serum; MEM, minimal Eagle's medium; TLC, thin-layer chromatography; GSLs, glycosphingolipids; PBL, peripheral blood lymphocytes.

(Boyum, 1968) at a force of 400*g* at the plasma–F-H interphase. Mononuclear cells were collected from the plasma–F-H interphase and the combined cell suspension was diluted 1:3 in phosphate-buffered saline (PBS, 0.02 M $PO_4$, 0.15 M NaCl, pH 7.4) and centrifuged at 300*g* for 10 min at 4 °C. The cells were washed three times in PBS by centrifugation at 250*g* for 10 min at 4 °C and finally resuspended in 10 mL of PBS. Aliquots of the cell suspension were removed for cell counts, differential cell counts (see below), protein determination (by the microbiuret method of Itzhaki and Gill, 1964), and B cell determination (see below). The remaining cell suspension was centrifuged at 100*g* for 10 min at 4 °C, the supernatant was removed by suction, and the cell pellet was stored at −20 °C for subsequent GSL analysis.

*Tonsil Lymphocytes.* Freshly removed human tonsils were finely minced in MEM containing 10% fetal calf serum (FCS) and the suspension was filtered through a funnel containing a loosely packed plug of glass wool that had been prewashed in MEM containing 10% FCS. The cells were centrifuged at 300*g* for 10 min at 4 °C, resuspended in 10 mL of the same medium, counted, and diluted to $4 \times 10^8$ leukocytes per 20 mL, and aliquots of 20 mL of cells were centrifuged in 10 mL of F-H. The mononuclear cells were processed as above for PBL.

*Thymocytes.* Freshly excised human thymus, obtained from thymuses removed during cardiac surgery, was processed in the same manner as human tonsils except that aliquots of the washed cells were not removed for protein determination and the F-H step was eliminated if the original suspension contained fewer than 1% erythrocytes.

*Differential Cell Counts.* An aliquot of washed cells (0.1 mL) was added to 0.5 mL of serum (FCS or autologous serum) in a 12 × 75 mm glass tube which was then centrifuged at 100*g* for 30 s. The supernatant serum was decanted and the cell pellet resuspended in approximately 0.2 mL of serum. Smears were prepared on glass slides and the cells were air dried and fixed in 100% methanol. The cells were stained with hematoxylin (Hematoxylin-Harris, Harleco No. 638) followed by OG-6 (Papanicolaou Stain, OG-6, Harleco No. 7052x) and mounted with Permount (Fisher Scientific, No. SO-P-15). Differential counts were performed using a Zeiss Universal microscope equipped with a 100× oil immersion objective lens and the percentages of plasma cells, monocytes, granulocytes, lymphocytes, and erythrocytes were determined on the basis of counting 500 cells per slide.

*Surface Immunoglobulin Staining.* Surface immunoglobulin staining of lymphocytes was performed as a marker of B cells by direct immunofluorescence using the method of Preud'homme and Seligmann (1972) as described in Stein-Douglas et al. (1976).

### *B. Glycolipid Extraction and Purification*

All solvents used were of reagent grade and were redistilled (Fisher Chemicals or Mallinckrodt Chemical Works), and mixtures of solvents were prepared on a volume/volume basis. The lipids extracted from all cell sources were fractionated on DEAE-Sephadex, as indicated in Figure 1, to separate neutral GSLs from gangliosides. In the initial phase of the work with PBLs, DEAE Fr. I was saponified and the neutral GSLs were purified by chromatography on a column of Unisil (scheme I). The neutral GSLs of tonsils and thymocytes were purified by scheme II in which DEAE Fr. I is acetylated and fractionated on Florisil by the method of Saito and Hakomori (1971). Scheme II is preferable because it permits separation of sphingomyelin and plasmalogens from neutral GSLs.

*Lipid Extraction.* Frozen cell pellets were emptied into a 500-mL stainless steel Sorvall homogenizing container and the tubes containing the pellets were rinsed with distilled water. The final volume of the pellet (or combined pellets) plus the rinses was brought up to 10 mL. The cells were extracted with 19 volumes (190 mL) of chloroform–methanol (C–M, 1:1) at room temperature, homogenized in a Sorvall Omni-Mixer (Ivan Sorvall Inc.), and the suspension was centrifuged at 1000*g* for 30 min. The supernatant was saved and the pellet was reextracted by the addition of 10 mL of distilled water and 19 volumes of C–M (1:2). The two supernatants were combined, evaporated to dryness at 40 °C on a rotary vacuum evaporator, redissolved in 10 mL of C–M (1:1) using heat (37 °C) and low power sonication with a bath-type sonicator (Heat Systems-Ultrasonics, Inc.), and filtered through a sintered glass filter. The residue was washed twice with 5 mL of C–M (1:1) and the combined filtrates were evaporated to dryness and redissolved in 10 mL of C–M (1:1); this material will be referred to as the C–M extract.

*Desalting.* Lipid fractions to be desalted were evaporated to dryness on a rotary evaporator and redissolved in 10 mL of C–M (1:1) using heat and sonication. Chloroform was added to bring the ratio of C–M to 2:1. The solution was cooled for a minimum of 2 h at 4 °C and filtered through a sintered glass filter, and the original flask, filter, and salt cake were rinsed three times with 1 mL of cold C–M (2:1).

*DEAE Chromatography.* DEAE chromatography was performed on Sephadex A-25 (Pharmacia Fine Chemicals) by the method of Ledeen et al. (1973) except that 1.1 g of DEAE was used and the sample was applied to the column in 98 mL of M–C–$H_2O$ (60:30:8). The first fraction (Fr. I) consisted of 148 mL of M–C–$H_2O$ (60:30:8) (sample volume +50 mL) and the second fraction (Fr. II) was eluted with 98 mL of M–C–0.8 M sodium acetate (60:30:8).

*Saponification:* Method I. A dried lipid fraction was treated with 1 mL of 0.2 N KOH in methanol +1 mL of $CHCl_3$, sonicated, and incubated at 37 °C for 3 h. The reaction was stopped by the addition of 1 mL of aqueous 0.2 N HCl in the cold and the mixture was dialyzed against distilled water for 2 days at 4 °C.

Method II. A dried lipid fraction was treated with 10 mL of 0.1 N NaOH in methanol and incubated at 37 °C for 3 h. The reaction mixture was acidified to pH 2 by dropwise addition of 1 N HCl in an ice bath, and after addition of 10 mL of cold distilled water the contents of the flask were lyophilized.

*Unisil Chromatography:* Method I. A column was prepared from 5 g of Unisil (activated silicic acid, 200–325 mesh, Clarkson Chemical Co., Inc.) that had been activated overnight at 120 °C. The column was packed in diethyl ether (approximately 10 mL) and was washed with 100 mL of $CHCl_3$. The sample was applied to the column in 10 mL of $CHCl_3$, followed by two 5-mL rinses of $CHCl_3$. The first fraction consisted of 100 mL of $CHCl_3$ (including the sample volume), the second fraction of 90 mL of C–M (1:1), and the third of 100 mL of C–M (2:8).

Method II. A column was prepared in $CHCl_3$ from 3 g of Unisil 200–325 mesh (not reactivated). The packed Unisil column was washed with 100 mL of $CHCl_3$ (including the sample volume). The second fraction consisted of 300 mL of C–M (1:1), including three 5-mL aliquots used to rinse the original sample flask in order to dissolve any lipid which was insoluble in $CHCl_3$. The three rinses were applied to the column separately before the remainder of the Fr. II solvent.

*Florisil Chromatography.* Florisil chromatography of acetylated lipids was carried out by the method of Saito and Hakomori (1971) using 5 g of 60–100 mesh Florisil (Fisher

TABLE I: Structures of Glycosphingolipids.[a]

| | |
|---|---|
| A. Neutral glycolipids | |
| Glucosylceramide (CMH) | Glc-Cer |
| Lactosylceramide (CDH) | Gal(β,1 → 4)Glc-Cer |
| Trihexosylceramide (CTH) | Gal(α,1 → 4)Gal(β,1 → 4)Glc-Cer |
| Globoside | GalNAc(β,1 → 3)Gal(α,1 → 4)Gal(β,1 → 4)Glc-Cer |
| Paragloboside | Gal(β,1 → 4)GlcNAc(β,1 → 3)Gal(β,1 → 4)Glc-Cer |
| Forssman | GalNAc(α,1 → 3)GalNAc(β,1 → 3)Gal(α,1 → 4)Gal(β,1 → 4)Glc-Cer |
| B. Gangliosides | |
| $G_{M3}$ (hematoside) | Gal(β,1 → 4)Glc-Cer<br>3<br>↑ α<br>2<br>NeuNAc |
| $G_{M2}$ (Tay-Sachs) | GalNAc(β,1 → 4)Gal(β,1 → 4)Glc-Cer<br>3<br>↑ α<br>2<br>NeuNAc |
| Asialo $G_{M1}$ | Gal(β,1 → 3)GalNAc(β,1 → 4)Gal(β,1 → 4)Glc-Cer |
| $G_{M1}$ | Gal(β,1 → 3)GalNAc(β,1 → 4)Gal(β,1 → 4)Glc-Cer<br>3<br>↑ α<br>2<br>NeuNAc |
| $G_{D1a}$ | Gal(β,1 → 3)GalNAc(β,1 → 4)Gal(β,1 → 4)Glc-Cer<br>3 3<br>↑ α ↑ α<br>2 2<br>NeuNAc NeuNAc |
| $G_{D1b}$ | Gal(β,1 → 3)GalNAc(β,1 → 4)Gal(β,1 → 4)Glc-Cer<br>3<br>↑ α<br>2<br>NeuNAc(α,8 ← 2)NeuNAc |
| $G_{T1}$ | Gal(β,1 → 3)GalNAc(β,1 → 4)Gal(β,1 → 4)Glc-Cer<br>3 3<br>↑ α ↑ α<br>2 2<br>NeuNAc NeuNAc(α,8 ← 2)NeuNAc |
| Sialosyl paragloboside | NeuNAc(α,2 → 3)Gal(β,1 → 4)GlcNAc(β,1 → 3)Gal(β,1 → 4)Glc-Cer |

[a] Gal, D-galactose; Glc, D-glucose; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; NeuNAc, *N*-acetylneuraminic acid; Cer (ceramide), *N*-acylsphingosine.

Scientific Co.) for each column. The sample and fraction volumes were modified as follows: the sample was applied to the column in 4 mL of hexane–dichloroethane (H-DCE) (1:4) + three 2-mL rinses (H-DCE, 1:4). The first fraction consisted of 50 mL of H-DCE (1:4) (including the sample volume), the second of 50 mL of DCE, third fraction 40 mL of DCE–acetone (1:1), and the fourth fraction 50 mL of DCE–methanol–water (2:8:1).

*Deacetylation.* Dried samples were deacetylated with 0.2 mL of 2 N KOH in methanol for 2 h at room temperature. The reaction flask was transferred to an ice bath and 5 mL of cold distilled water was added, followed by 0.3 mL of aqueous 0.2 N HCl. The mixture was then frozen and lyophilized.

*Neuraminidase Treatment.* Dried ganglioside samples (containing approximately 10 μg of neuraminic acid) were dissolved in 0.5 mL of 0.01 N acetic acid, pH 5.0, and 0.05 mL of neuraminidase (mucopolysaccharide *N*-acetylneuraminylhydrolase, EC 3.2.1.18, 500 units/mL, Behring Diagnostics) was added, and the solution was overlaid with 0.2 mL of toluene and incubated at 37 °C for 24 h with additions of 0.025 mL of neuraminidase to the mixture after 12 and 18 h of incubation. The reaction was stopped by the addition of 10 mL of C–M (2:1) and the mixture was centrifuged at 500*g* at 4 °C for 20 min. The supernatant was removed and evaporated to dryness under $N_2$, and the residue was redissolved in C–M (1:1) in the original volume of the ganglioside sample.

### C. Glycolipid Analysis

*Thin-Layer Chromatography.* Precoated silica gel 60 plates, 0.25 mm (E. Merck, E.M. Laboratories, Inc.) were pre-run in the chromatographic solvents and then activated at 110 °C for 45 min. The GSL standards used (Table I) were described previously (Naiki et al., 1974, 1975). Following application of the sample the plate was dried for 15 min under vacuum. Analytical TLC of neutral GSLs was carried out in C–M–$H_2O$ (60:35:8 or 60:30:5), and preparative TLC was performed with the former solvent. Following preparative TLC the samples were visualized with iodine vapor and were scraped off the plate, leaving approximately 0.25 cm of each band on the plate. The residual sample, and a mixture of standard compounds that was chromatographed on one end of the same plate, was stained with α-naphthol (Siakotos and Rouser, 1965) and positive bands were eluted from the silica gel with C–M–$H_2O$ (50:50:10). Gangliosides were chromatographed in C–M–2.5 N $NH_4OH$ (60:40:9) and visualized with resorcinol (Svennerholm, 1957).

*Gas Chromatography.* Gas chromatographic analyses of trimethylsilyl derivatives of sugars and fatty acid methyl esters

TABLE II: Cell Yields and Protein Analysis.

| Tissue | No. preparations in pool | No. cell[a] donors | Total No. cells | Total[b] protein (mg) | mg of protein per $10^8$ cells |
|---|---|---|---|---|---|
| Thymus | 2 | 2 | $2.1 \times 10^9$ | ND[c] | ND |
| PBL | 3 units of blood | 1 | $3.6 \times 10^8$ [d] | 11.6 | 3.2 |
| Tonsil | 3 | 14 | $1.3 \times 10^{10}$ | 356.0 | 2.8 |

[a] That is, number of individual tonsils or thymuses. [b] Determined on each preparation in the pool. [c] ND, not determined. [d] Not representative of the possible yield as some cells were lost during this preparation.

TABLE III: Differential Counts of Cell Preparations.[a]

| Preparation | Plasma cells | Mononuclear phagocytes | Granulocytes | Erythrocytes | Mononuclear cells[b] |
|---|---|---|---|---|---|
| Thymus | 0 | <0.05 | 0.2 | 0.6 | 99 |
| PBL | 0 | 2 | 0.2 | 1 | >96 |
| Tonsil | <0.1 | 1.5 | 0.2 | 0.9 | >96 |

[a] The numbers represent the average percentage of cells in each pool. Determinations were made on each preparation by counting 500 cells. [b] Lymphocytes or thymocytes.

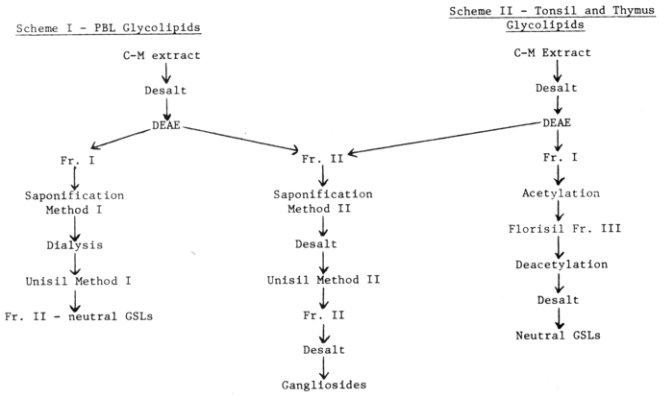


FIGURE 1: Outline of procedures for purification of glycosphingolipids.

were performed by modifications of the method of Vance and Sweeley (1967) described previously (Naiki et al., 1975; Marcus et al., 1976). Sialic acid was measured by the method of Yu and Ledeen (1970).

## Results

*Cell Preparations.* Table II shows the yields and protein content of the cell preparations used in the glycolipid analysis. Cell yields were approximately $1 \times 10^6$ lymphocytes per mL of blood and $10^9$ lymphocytes per tonsil. Table III contains data on the purity of the cell preparation.

The percent of B lymphocytes, cells that exhibited cell-surface immunoglobulin, was <0.5% in the thymocyte preparation, 12% in the PBL preparation, and 51% in the tonsil lymphocyte preparation.

*Neutral Glycolipids.* The neutral glycolipids prepared by schemes I and II (Figure 1) from different tonsil preparations appeared to be similar, but the material prepared by scheme I contained nonglycolipid contaminant. This contaminant migrated on TLC with ceramide trihexoside (CTH) and was propably sphingomyelin. The acetylation procedure used in scheme II separated sphingomyelin from the GSLs. The neutral GSLs purified by scheme I from PBL, and by scheme II from thymocytes and tonsil lymphocytes, are compared in Figure 2. All three tissues have detectable bands that comigrate with CMH and CDH, but no band is present in the CTH region in PBL and only a very faint spot is seen in thymus. In

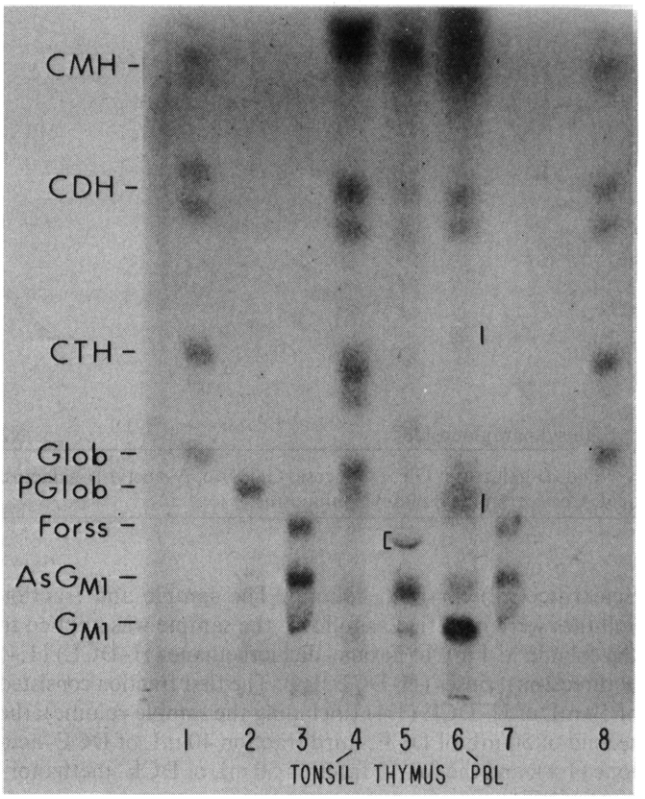


FIGURE 2: Neutral glycolipids of tonsil lymphocytes, PBL, and thymocytes. Thin-layer chromatogram of tonsil lymphocyte, peripheral blood lymphocyte (PBL), and thymocyte neutral glycolipids. Lanes 1, 2, 3, 7, and 8 contained standard compounds; lane 4, tonsil neutral glycolipids; lane 5, thymocyte neutral glycolipids; lane 6, PBL neutral glycolipids. Abbreviations not listed in Table I: PGlob, paragloboside; Forss, Forssman; and $AsG_{M1}$, asialo $G_{M1}$. The amount of tonsil GSLs applied to the plate was equivalent to the yield from $9.8 \times 10^8$ cells; thymocyte GSLs were equivalent to the yield from $4.1 \times 10^8$ cells; and PBL neutral glycolipids were equivalent to the yield from $1.1 \times 10^8$ cells. Silica gel 60 plate was developed in C–M–$H_2O$ (60:30:5) and sprayed with $\alpha$-naphthol. All bands were purple except those indicated with a line in lane 6, which were yellow. The band marked with a bracket in lane 5 was identified as a ganglioside.

contrast, tonsil lymphocytes have an appreciable amount of $\alpha$-naphthol-positive material that comigrates with CTH. A faint band is seen in the globoside region of PBL and no $\alpha$-naphthol-positive bands were seen in the globoside and para-

TABLE IV: Ratio of Sugars in Tonsil Lymphocyte Neutral Glycolipids.

| | Molar ratios[a] | | | | |
|---|---|---|---|---|---|
| | Globoside standard | Band 8[b] CMH | Band 7 CDH | Band 6 CTH | Band 5 globoside |
| Glucose | 1 | 1 | 1 | 1 | 1 |
| Galactose | 2.0 | | 1.06 | 1.76 | 1.49 |
| *N*-Acetylgalactosamine | 1.0 | | | | 0.43 |

[a] Corrected for detector response. [b] Band numbers refer to preparative TLC designations.

TABLE V: Fatty Acid Composition of Tonsil Lymphocyte Glycolipids.[a]

| Fatty acid | CMH | CDH | CTH | Globoside |
|---|---|---|---|---|
| $C_{16:1}$ | b | | | 3 |
| $C_{16:0}$ | | | 16 | 19 |
| $C_{17:0}$ | 7 | 11 | | |
| $C_{18:1}$ | 3 | 8 | 7 | 5 |
| $C_{18:0}$ | 17 | 22 | 20 | 9 |
| $C_{20:0}$ | 14 | 8 | 7 | 5 |
| $C_{22:1}$ | | | 3 | 3 |
| $C_{22:0}$ | 25 | 20 | 7 | 7 |
| $C_{24:1}$ | 26 | 23 | 30 | 41 |
| $C_{24:0}$ | 8 | 8 | 6 | 7 |
| $C_{25:0}$ | | | 4 | |

[a] The results are expressed as the % of total for each glycolipid. Fatty acids not detected in any of the glycolipids are not listed. [b] Not detected.

globoside regions of the thymus sample. Two bands that migrated more slowly than globoside were observed in the PBL and thymus samples and in trace amounts in the tonsil. The narrow band seen in the thymus sample (marked in Figure 2) was identified as a resorcinol-positive band which comigrated with a hematoside standard. A trace of the same resorcinol-positive band found the thymus was detected in the PBL sample, although it is poorly visible due to the presence of the contaminant.

The tonsil neutral glycolipids were fractionated by preparative TLC and all $\alpha$-naphthol-positive bands were analyzed for sugars, fatty acids, and long chain bases. The slowly migrating bands and the band that comigrated with paragloboside were present in insufficient quantities for analysis. The ratios of sugars in bands 5–8 (Table IV) are consistent with their identification as globoside, CTH, CDH, and CMH, respectively. The yields of individual GSLs in nanomoles/1 $\times 10^8$ tonsil lymphocytes were: CMH, 1.23; CDH, 0.46; CTH, 0.54; and globoside, 0.77. The relative molar proportions of each of the four GSLs were CMH, 42%, CDH, 16%, CTH, 18%, and globoside, 24%.

*Long-Chain Base Analysis.* The amount of glycolipid obtained from the tonsil preparation was not sufficient to perform a separate hydrolysis for individual long chain base identification. GLC analysis of $Me_4Si$ derivatives on 3% OV-1 run at 210 °C revealed peaks (predominantly $C_{18}$ and $C_{20}$) with the same retention times as long chain base peaks in the standards, globoside, Tay-Sachs, and bovine brain gangliosides (see Vance and Sweeley, 1967).

*Fatty Acid Analysis.* The fatty acid composition of the neutral glycolipids is given in Table V; hydroxy fatty acids were not detected.

*Gangliosides.* The gangliosides of tonsil lymphocytes, PBL, and thymocytes are compared by TLC in Figure 3. The most prominent band seen in the tonsil preparation migrates more slowly than bovine brain hematoside, but it comigrates with human spleen hematoside (data not shown). Identification of this band as hematoside was supported by the results of neuraminidase treatment of an aliquot of the tonsil lymphocyte ganglioside fraction. The major band disappeared following neuraminidase treatment and a new band appeared that comigrated with a CDH standard. Tonsil lymphocytes contain several other gangliosides that do not comigrate with bovine brain ganglioside standards but they do comigrate with human spleen gangliosides. In addition to hematoside, one of the slower migrating gangliosides was sensitive to neuraminidase (data not shown).

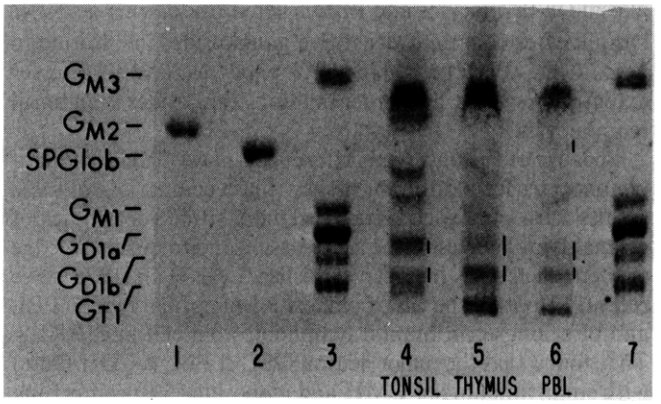


FIGURE 3: Gangliosides of tonsil lymphocytes, PBL, and thymocytes. Thin-layer chromatogram of tonsil lymphocyte, PBL, and thymocyte gangliosides. Lane 1, Tay-Sachs ($G_{M2}$) ganglioside standard; lane 2, SPG, sialosylparagloboside standard; lanes 3 and 7, bovine brain gangliosides and $G_{M3}$ standards; lane 4, tonsil gangliosides; lane 5, thymus gangliosides; and lane 6, PBL gangliosides. The quantities of gangliosides applied to the plate were 3.8 $\mu$g of sialic acid for the tonsils (equivalent to yield from $1.3 \times 10^9$ cells), 3.5 $\mu$g sialic acid for the thymocytes ($1 \times 10^9$ cells), and 2.3 $\mu$g sialic acid for the PBL ($1.5 \times 10^8$ cells). Silica gel 60 plate was developed in C–M–2.5 N $NH_4OH$ (60:40:9) and sprayed with resorcinol. All bands were purple except those indicated by lines in lanes 4, 5, and 6, which were yellow.

Inasmuch as the gangliosides were not present in sufficient quantities to analyze individual components, the lipid-bound sialic acid was determined as an indication of the total amount of ganglioside present. The highest concentration of lipid-bound sialic acid was found in PBL, which contained 1.59 $\mu g/10^8$ lymphocytes, and tonsils and thymocytes contained 0.29 and 0.50 $\mu g/10^8$ cells, respectively.

## Discussion

These studies clearly indicate that both qualitative and quantitative differences in GSL composition exist between highly purified lymphocytes obtained from different human tissues. The GSLs of tonsil lymphocytes differ significantly from both thymocytes and PBL. Although sufficient material was present for quantitation of individual GSLs by GLC only in the tonsil sample, some approximations can be made by visual examination of the thin-layer plates as to the relative amounts of different glycolipids. Thymocytes are relatively poor in CDH and CMH, and PBL are relatively rich in these GSLs compared with tonsil lymphocytes. Both thymocytes and PBL contain large amounts of slowly migrating neutral GSLs but only traces of these compounds were observed in tonsil lymphocytes. Most striking is the virtual absence of CTH in thymocytes and PBL compared with tonsil lymphocytes. The ganglioside compositions of thymocytes and PBL are similar and differ from that of tonsil lymphocytes. The major gan-

glioside in thymocytes and PBL is hematoside, whereas tonsil lymphocytes contain at least five gangliosides in addition to hematoside. Peripheral blood lymphocytes, and to a lesser extent thymocytes, are richer in total gangliosides than tonsil lymphocytes.

Because of the observed differences in the GSL content of lymphocytes from different tissues, direct comparison of these results with published data must be confined to studies of normal lymphocytes of the same tissue, and to our knowledge only one such study has been published. Levis et al. (1976) have recently reported the GSL composition of normal human PBL and of a long-term human lymphoblastoid cell line, BRI 8. They found that the major neutral GSL of PBL is CDH (90%) with small amounts of CMH and globoside (5%) comprising the remainder of the neutral GSL fraction. In the analytical TLC presented here (Figure 2) CDH does not appear to comprise 90% of the GSL in PBL, but quantitative measurements have not been made. Our results are in agreement in finding no CTH and relatively little globoside in PBL. The authors do not comment on the presence or absence of slowly migrating neutral GSLs, but it is possible that these glycolipids were missed because they partition into the aqueous phase during Folch partitioning (Folch et al., 1957; Hakomori and Watanabe, 1976), and Levis et al. (1976) examined only the chloroform–methanol phase for the presence of neutral GSLs. In agreement with the results reported here, they found only hematoside in PBL, but their results differ with those presented here in the quantity found. Our data show that there is 0.5 $\mu$g of lipid-bound sialic acid/mg of protein in PBL, which is equivalent to approximately 2 $\mu$g of hematoside; but Levis et al. (1976) found only 0.05 $\mu$g of hematoside/mg of protein in PBL. This discrepancy could be due to the fact that they measured only the Folch aqueous phase hematoside, and not the hematoside that partitions into the organic phase.

One study of the neutral GSLs of leukemic lymphocytes has been published (Hildebrand et al., 1971). Chronic lymphocytic leukemia cells were found to contain only CMH, and acute lymphoblastic leukemia cells contained both CMH and CDH in an average composition of 30% and 70%, respectively. Hildebrand et al. (1972) found an average of 0.002 $\mu$g of lipid-bound sialic acid/mg of protein in chronic lymphocytic leukemia cells.

A major finding of this study is the similarity between thymocyte and PBL neutral GSLs and gangliosides, and their difference from tonsil lymphocytes. Thymocytes contain no B cells, PBL have approximately 10% and tonsils 50% B cells. The CTH and globoside in tonsil lymphocytes could be due to the presence of a higher concentration of B cells than in thymocytes and PBL. Levis et al. (1976) reported that the human lymphoblastoid cell line BRI 8 contained only $^1/_2$ to $^1/_3$ as much total GSL as PBL, but the proportion of CTH increased from nondetectable in PBL to 70% in BRI 8. Almost all human lymphoblastoid cell lines are B cell lines, as determined by the presence of cell surface immunoglobulin or immunoglobulin synthesis (Evans et al., 1974), and BRI 8 has been reported to be a B-cell line (Steel et al., 1974). Recently Naiki and Marcus (1975) have demonstrated that the human blood group antigen $P^k$ is CTH. Fellous et al. (1974) used human antibodies to $P^k$ to study the expression of this antigen on PBL from 10 normal donors, the lymphoblastoid cell lines derived from three of the donors, and two commercially available lymphoblastoid lines. They found that the $P^k$ antigen was not detectable in PBL but was detectable in all five of the cell lines. One of the cell lines they used (RPMI 8866) has been shown to be a B cell line (Evans et al., 1974) and the others can be assumed to be B cell lines. In a more comprehensive study of $P^k$ expression on lymphoid cells, Fellous et al. (1977) reported the following pattern of $P^k$ expression; ($P^k$-negative cells) T-lymphoblastoid lines, acute lymphoblastic leukemias (of T cell or null cell origin), CLLs of T cell origin, PBLs and enriched PBL T cells; ($P^k$-positive cells) B-lymphoblastoid lines, CLLs of B cell origin, tonsil lymphocytes, and enriched PBL B cells. Recently Schwarting and Marcus (1977) have studied the reactivity of purified antibodies to CTH with PBLs and they found that only peripheral blood B cells and not T cells reacted with this antibody.

We are now studying the reaction of normal human lymphocytes with antibodies to six GSLs, and we plan to perform immunochemical analyses of lymphocytes from patients with lymphoproliferative and immunodeficiency diseases. These studies should provide new serological markers for identification of lymphocyte subpopulations, and define alterations in GSL content and antigenic expression in abnormal lymphocytes.

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# Absolute Configuration of Tritiated *O*-Alkylglycerol Synthesized Enzymatically from [1,3-$^3H_2$, 1,3-$^{14}C_2$]Dihydroxyacetone Phosphate†

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ABSTRACT: *O*-Alkyldihydroxyacetone phosphate is synthesized enzymatically from hexadecanol and acyldihydroxyacetone phosphate. In this process there is a hydrogen exchange in which the *pro-R* hydrogen of C-1 of the *sn*-glycerol moiety is lost. This hydrogen is replaced by a hydrogen from the medium. In order to obtain additional information on the mechanism of ether bond formation, it would be of interest to know whether or not the hydrogen exchange results in a change of configuration in the product, *O*-alkyldihydroxyacetone phosphate. By using *O*-alkylglycerol prepared both chemically and enzymatically from isomerase-treated [1,3-$^3H_2$, 1,3-$^{14}C_2$]dihydroxyacetone phosphate and an *O*-alkylglycerol cleavage enzyme system, it was shown that the hydrogen exchange occurs with retention of configuration of the substituents of C-1 of the *sn*-glycerol moiety.

The conversion of acyldihydroxyacetone phosphate to *O*-alkyldihydroxyacetone phosphate is an important step in glycerol ether biosynthesis (Hajra, 1970; Wykle et al., 1972). In this enzymatic reaction, the fatty acid moiety of acyldihydroxyacetone phosphate is lost and a fatty alcohol is gained, resulting in the formation of *O*-alkyldihydroxyacetone phosphate. We have previously shown that there is a simultaneous stereospecific loss of hydrogen from carbon one of the dihydroxyacetone phosphate of acyldihydroxyacetone phosphate (Friedberg et al., 1971). The hydrogen which is lost is the same one which is exchanged in the triosephosphate isomerase reaction (Friedberg et al., 1972). The dihydroxyacetone phosphate moiety then gains a hydrogen from the medium (Friedberg and Heifetz, 1975). Thus when acyldihydroxyacetone phosphate, hexadecanol, and Ehrlich ascites tumor cell microsomes are incubated in the presence of tritiated water, the *O*-alkyl lipid formed becomes tritiated. A question which is immediately suggested is whether or not the configuration of the substituents of carbon one of the dihydroxyacetone phosphate of *O*-alkyldihydroxyacetone phosphate retains the same configuration as the substrate dihydroxyacetone phosphate. The answer to this question would be important in determining the mechanism of ether bond formation.

In the present investigation we demonstrate that the hydrogen exchange occurs with retention of configuration at carbon one of *sn*-glycerol of *O*-alkylglycerol. Thus, the *pro-R* hydrogen is initially lost and is replaced by a hydrogen from the medium in the *pro-R* position.

Davis and Hajra (1977) recently presented data which confirm that there is a hydrogen exchange in the conversion of acyldihydroxyacetone phosphate to *O*-alkyldihydroxyacetone phosphate. These workers also indicate that they have preliminary data suggesting that this occurs with retention of configuration.

## Materials and Methods

[1,3-$^3H_2$]Dihydroxyacetone phosphate and [1,3-$^{14}C_2$]-dihydroxyacetone phosphate were synthesized as previously described except that triethanolamine was used instead of glycine in the buffer solution (Friedberg et al., 1971).

The preparation of Ehrlich ascites tumor cells and *Tetrahymena pyriformis* microsomes was carried out as previously described (Friedberg et al., 1971; Friedberg and Heifetz, 1975).

*Biosynthetic Preparation of O-Alkyl[S-1-$^3H_1$, 3-$^3H_2$, 1,3-$^{14}C_2$]-sn-glycerol.* A mixture of [1,3-$^3H_2$]- and [1,3-$^{14}C_2$]-dihydroxyacetone phosphate, 33.12 μCi and 2.24 μCi, respectively, with an absolute ratio of 14.8 (0.098 μmol, total) was incubated at 37 °C with 16 mL (4 mg of protein per mL) of Ehrlich ascites tumor cell microsomes in a final volume of 21.76 mL. The mixture contained: ATP, 7.35 mM; magnesium

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