Quantitative analysis of neutral glycosphingolipids from human lymphocyte subpopulations

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The glycosphingolipids of normal human lymphocytes from individual donors were analysed by high-pressure liquid chromatography. In addition, purified T- and B-lymphocytes were examined separately. Lactosylceramide was shown to be the major neutral glycosphingolipid in human lymphocytes, and monohexosylceramide, trihexosylceramide, globoside and paragloboside were all detected in smaller amounts. Analysis of purified B- and T-cell fractions revealed that each of these populations contained a similar qualitative profile for neutral glycosphingolipids, but that quantitatively, B-cells contained several times more of each glycosphingolipid per cell than did T-cells.

Human lymphocytes are thought to be composed of several subpopulations, each having specialized functional properties (Evans *et al.*, 1978; Reinherz & Schlossman, 1979; Reinherz *et al.*, 1979). Many of these subpopulations have been identified by antisera, but still very little is known about the chemical composition of these distinct functional subsets of lymphocytes.

Glycosphingolipids are major lipid constituents of the cell surface membrane of all mammalian cells (Hakomori, 1975). The sugar moieties of many glycosphingolipids are exposed on the cell surface and are known to be antigenically active (Marcus & Schwarting, 1976). Glycosphingolipids have a unique composition in different functional cell types, and have been shown to be altered qualitatively and quantitatively when cells are transformed (Hakomori *et al.*, 1972; Hakomori, 1973, 1975; Fishman & Brady, 1976). Although there is very little evidence, glycosphingolipids are thought to function as mediators of cell–cell interactions and/or intracellular communication.

Until now it had not been possible to quantify accurately the glycosphingolipids obtained from small tissue samples, but by employing recent h.p.l.c. methodology, we report in the present paper the quantities of individual glycosphingolipids from as few as 5×10^6 purified human T- and B-lymphocytes.

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

Materials and methods

Isolation of lymphocytes

Human peripheral blood (50 ml) from normal healthy donors was drawn into heparinized tubes. The blood was layered over Ficoll-hypaque and spun at 400g for 40 min (Boyum, 1968). Lymphocytes were harvested at the interface and washed three times in RPMI-1640 tissue culture medium (Gibco, Grand Island, NY, U.S.A.) containing 10% foetal calf serum. The cells were diluted to 1×10^6 cells/ml and placed in plastic culture dishes (Falcon, Oxnard, CA, U.S.A.) for 30–45 min at 37°C (Ozer *et al.*, 1979). The non-adherent cells were removed from the dish, counted and either processed further or extracted with chloroform/methanol (2:1, v/v). Portions of cells were removed to determine the purity of the cell preparations before analysis.

Isolation of T-lymphocytes

After removal of the adherent cell population as described above, lymphocytes were incubated at a 1:50 ratio with sheep erythrocytes by the method of Kaplan *et al.* (1976). After rosetting, the cells were centrifuged on Ficoll-hypaque, and the upper fractions were removed, leaving the rosetted cells. The sheep erythrocytes were then lysed by addition of NH_4Cl at 37°C for 5 min (Ozer *et al.*, 1979). The lysed sheep erythrocytes were then removed by three washings in 0.02 M-phosphate-buffered saline

(pH 7.2). The remaining T-lymphocytes were counted, examined for purity and extracted.

Isolation of B-lymphocytes

After removal of the adherent cell population as described above, lymphocytes were washed three times in phosphate-buffered saline and incubated at 37°C for 10–15 min at 2×10^6 cells/ml. Sheep erythrocytes coated with complement components ('EAC sheep erythrocytes'; Cordis, Miami, FL, U.S.A.) were incubated at 37°C for 10–15 min at a concentration of 10^9 cells/ml (Ehlenberger & Nussenzweig, 1976). Equal volumes of lymphocytes and EAC sheep erythrocytes were added together and incubated at 37°C for 45–60 min. This preparation was then layered on Ficoll-hypaque and centrifuged at 400g for 40 min. The B-cell rosettes were obtained as above, lysed with NH_4Cl buffer, washed extensively, counted, examined for purity and extracted.

Purity of cell preparations

Three portions were removed from each cell preparation to assess its purity. One portion was incubated at 37°C for 45 min with $1.1 \mu\text{m}$ latex particles (Dow Diagnostics, Indianapolis, IN, U.S.A.). Cells that ingested one or more latex particles were considered to be phagocytic cells.

A second portion was incubated with fluorescein-conjugated goat anti-human immunoglobulin, (Fab' 2 fragments) (Cappel Labs, Cochranville, PA, U.S.A.) to enumerate B-cells, as previously described (Stein-Douglas *et al.*, 1976).

The third portion was used to prepare slides for differential cell counts. Smears were stained with haematoxylin followed by OG-6 as described previously (Stein & Marcus, 1977).

Extraction of glycosphingolipids

Washed cells were suspended in phosphate-buffered saline to which 20 ml of chloroform/methanol (2:1) was added. The internal standards *N*-acetylpsychosine and asialoganglioside G_{M_2} were added, after previous analysis indicated that these glycolipids were not present in the cells, and the suspension was agitated for 1 h. The suspension was filtered and re-extracted with 20 ml of chloroform/methanol (2:1) for 2 h at 60°C. To the combined extracts was added 8 ml of water. After vigorous shaking a partition formed and the upper aqueous phase containing gangliosides was removed. The partition was repeated with the addition of 19.25 ml of methanol/water (1:1), as previously described (Folch *et al.*, 1957).

The lower phase was dried and placed on a 0.04 g silicic acid column (Unisil; Clarkson Chemical Corp.) in 1.0 ml of chloroform. The column was washed with another 1.0 ml of chloroform and 1.0 ml

of ethyl acetate before elution of the neutral glycolipid fraction with 4.0 ml of acetone/methanol (9:1).

This fraction was dried and treated with methanolic 0.6 M-NaOH at room temperature for 1 h, then neutralized with HCl. The purified glycosphingolipid fraction was recovered from this reaction mixture by solvent partition.

The dried glycolipids were benzoylated, as previously described, with 10% benzoyl chloride in pyridine (Ullman & McCluer, 1977).

High-pressure liquid chromatography

The h.p.l.c. (Waters Associates, Milford, MA, U.S.A.) analysis was carried out as previously described (Ullman & McCluer, 1978) on a Zipax (E.I. Dupont de Nemours, Inc., Wilmington, DE, U.S.A.) column (2.1 mm $\times$ 50 cm) with a 13 min linear gradient of 1–20% dioxan in hexane. U.v. detection at 230 nm was used.

Results

All cell preparations were individually characterized by several methods to judge their purity. Before extraction, three portions were removed. One portion was incubated with latex particles to examine the phagocytic properties of the cell preparations. A second portion was incubated with fluorescein-labelled anti-immunoglobulin to enumerate B-cells, and the third portion was used for differential cell counts. By using these criteria, unseparated lymphocytes consisted of between 5 and 20% B-cells and greater than 85% lymphocytes. T-cell preparations were greater than 97% immunoglobulin-negative non-phagocytic lymphocytes, whereas B-cell preparations were in each case greater than 85% immunoglobulin-positive non-phagocytic lymphocytes.

Fig. 1(b) is a plot of 12 purified glycolipid standards, chromatographed together. All glycolipids chromatograph as single peaks except for galabiosylceramide, which is represented by peaks 3 and 4. In addition, except for glucosyl- and galactosyl-ceramide, which co-chromatograph as peak 1, all glycolipids are easily separable and identifiable in this system.

Fig. 1(a) is a plot of the neutral glycosphingolipids from 0.58×10^7 lymphocytes from a normal donor. Peaks that correspond to peaks 2 and 7 of the standard represent the internal standards *N*-acetylpsychosine and asialoganglioside G_{M_2} respectively, known amounts of which were inserted at the beginning of the extraction procedure. The recoveries of these two standards were calculated and used as indicators to quantify the yield of lymphocyte glycolipids during the isolation procedure and the procedure for formation of deri-

Fig. 1. H.p.l.c. of neutral glycosphingolipids from (a) unseparated human peripheral blood lymphocytes and (b) standard compounds

Peaks are identified as the derivatives of: 1, glucosyl- or galactosyl-ceramide; 2, *N*-acetylpsychosine; 3 and 4, galabiosylceramide; 5, lactosylceramide; 6, globotriaosylceramide; 7, gangliotriaosylceramide; 8, lactotetraosylceramide; 9, globotetraosylceramide; 10, gangliotetraosylceramide; 11, neolactotetraosylceramide; 12, Forssman glycolipid.

vatives. *N*-Acetylpsychosine was chosen to represent the recoveries of the less polar glycosphingolipids, monohexosylceramide and lactosylceramide, whereas asialoganglioside G_{M2} was to represent the recoveries of the more polar lipids; trihexosylceramide, globoside and paragloboside. The recoveries of these two standards were always nearly equal, averaging 60%. The Folch partitions and silicic acid chromatography accounted for the major losses, which appear to be linear at least for the two standards employed in this study. Results reported here for content of human lymphocyte glycosphingolipids are corrected for the loss of internal standard from that individual experiment. The other five identifiable peaks in Fig. 1 relative to the standard are: 1, monohexosylceramide; 5, lactosylceramide; 6, trihexosylceramide; 9, globoside; 11, paragloboside (Table 1). These identifications are at present based on retention times relative to standards. Further chemical analysis will necessary to confirm these identifications.

In all individuals tested, lactosylceramide represents greater than 80% of the total neutral glycosphingolipids, and monohexosylceramide makes up about another 10% (Table 2a), which is in agreement with previously published results (Levis *et al.*, 1976). The three minor neutral glycosphingolipids; trihexosylceramide, globoside and paragloboside constitute together less than 10% of the total neutral glycosphingolipids in human lymphocytes. From

Table 1. Structures of glycosphingolipids
Abbreviation: Cer, ceramide.

Monohexosylceramide	GlcCer
Lactosylceramide	Gal(β1-4)GlcCer
Trihexosylceramide	Gal(α1-4)Gal(β1-4)GlcCer
Globoside	GalNAc(β1-3)Gal(α1-4)Gal(β1-4)GlcCer
Paragloboside	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcCer
Forssman	GalNAc(α1-3)GalNAc(β1-3)Gal(α1-4)Gal(β1-4)GlcCer

Table 2. Concentration of glycosphingolipids in normal human lymphocytes
Values are for single experiments for each individual donor.

		Content (pmol/10 ⁷ cells)				
(a)	Donor	Monohexosyl- ceramide	Lactosylceramide	Trihexosyl- ceramide	Globoside	Paragloboside
	1	245	1660	12	31	10
	2	239	1670	42	60	12
	3	92	876	23	92	15
	4	150	950	28	50	12
	5	93	1180	13	76	14
	Mean ± s.d.	164 ± 75.2	1267 ± 380	23.6 ± 12.3	61.8 ± 23.5	12.6 ± 2.0
(b)	4	136	914	28	25	10
	4	120	1030	14	45	20
	4	100	1180	44	84	12

Table 3. *Concentration of glycosphingolipids in separated human T- and B-lymphocytes*
 Values are for single experiments for each individual donor.

Donor	Content (pmol/10 ⁷ cells)				
	Monohexosylceramide	Lactosylceramide	Trihexosylceramide	Globoside	Paragloboside
T-cells					
1	158	146	21	9.0	7.0
2	33	184	3.7	4.6	1.9
3	56	242	6.1	18	2.4
4	70	532	2.7	4.3	2.1
5	101	130	10	13	6.8
Mean $\pm$ S.D.	83.6 $\pm$ 48.3	247 $\pm$ 165	8.5 $\pm$ 7.4	9.8 $\pm$ 5.8	4.0 $\pm$ 2.6
B-cells					
1	322	1620	62	66	49
2	172	3620	28	121	32
3	189	3860	64	246	30
4	245	2460	40	74	49
5	253	5400	87	119	31
Mean $\pm$ S.D.	236 $\pm$ 59.3	3392 $\pm$ 1441	56.2 $\pm$ 22.9	125 $\pm$ 72.1	38.2 $\pm$ 9.9

Table 2(a), it is apparent that there is considerable variation in absolute amounts of any one glycosphingolipid, so lymphocytes from one donor were examined several times over a 2 month period, and the results are shown in Table 2(b). The variations appeared to decrease as the quantities of each glycosphingolipid increased.

Table 3 shows the results of the glycolipid analysis of separated T- and B-lymphocytes. Again, there is considerable variation among individuals, but the ratio of one glycosphingolipid to another, within each cell type, remains quite constant. Lactosylceramide is the predominant glycosphingolipid in both T- and B-cells, and considerable amounts of monohexosylceramide are again detected. Again though, trihexosylceramide, globoside and paragloboside are minor components of both B- and T-cells.

In striking contrast to the qualitative similarities of T- and B-cell glycosphingolipids are the overwhelming quantitative differences seen in these two cell types. Table 3 shows that B-cells contain up to 10 or 12 times as much of several of these glycosphingolipids as do T-cells.

Discussion

The most critical factor in a study as this, an analysis of the chemical constituents of a subpopulation of cells, is to obtain the purest possible cell samples for analysis. We have therefore used the most recent developments in methodology to purify and characterize our cells as much as possible. Some methods, such as the use of iron carbonyl, were found to be ineffective, which in this case was replaced by the use of plastic tissue culture dishes to

remove monocytes. On the other hand, plastic has been shown to remove small numbers of B-cells, but what effect this potential loss may have on our results is not known.

Contamination of our cell preparations could come from several possible sources. A constant source of difficulty is monocytes. They have been depleted from all preparations, but their detection in purified cell samples remains controversial. Fluorescent labelled anti-immunoglobulin, which is used to detect B-cells, can also bind to monocytes. Differential staining can also yield ambiguous results when attempting to distinguish monocytes from lymphocytes, so the ingestion of latex particles was our best indicator of phagocytic cells.

T-cells were the most pure population that we could obtain. About a 30% yield was obtained by the rosetting procedure, but only preparations that were greater than 97% immunoglobulin-negative non-phagocytic cells were analysed. When unseparated peripheral blood lymphocytes were incubated on plastic dishes, a 50% recovery was obtained that consisted of greater than 85% non-phagocytic cells. EAC-rosetted B-lymphocytes were also 85% or better immunoglobulin-positive cells, incapable of ingesting latex particles.

A second source of contamination could result from the sheep erythrocytes that are used to rosette the T- and B-cells. Control experiments have shown that insignificant amounts of glycosphingolipids from sheep erythrocytes remain after lysis and washing with phosphate-buffered saline. Sheep erythrocytes contain only small amounts of the five glycosphingolipids reported here, but do contain very large amounts of another glycosphingolipid, the Forssman antigen. We have never been able to

detect any Forssman antigen in our human lymphocyte preparations. Therefore contamination from other sheep erythrocyte glycosphingolipids, which occur in much smaller amounts, seems unlikely.

A third source of exogenous glycosphingolipids could come from the foetal calf serum that is used in the cell culture medium. Serum is known to contain significant amounts of neutral glycosphingolipids and is used at a concentration of 10% in the medium. However, before extraction all cell preparations were washed three times in phosphate-buffered saline to remove these possible contaminants.

Of utmost significance is the sensitivity of this h.p.l.c. method, which allows for the analysis of less than 5×10^6 cells, and thus permits the completion of such a study. Previous analyses of human peripheral blood lymphocytes (Levis *et al.*, 1976; Stein & Marcus, 1977; Narasimham & Murray, 1978), which employed less sensitive techniques, required more than 10^8 cells while compiling no quantitative data and much less extensive qualitative results.

In studies reported in the present paper, 50 ml of blood was sufficient to examine B-cell glycosphingolipids, and considerably less was needed to obtain data on T-cells or unseparated lymphocytes. This provides a significant advance in terms of both time and accuracy over the previous method, which included preparative t.l.c. and gas chromatography of derivatives of glycosphingolipids.

Table 2 shows that there is considerable variation among individuals in their absolute amounts of any one glycosphingolipid. Values for one individual may be three times or higher than those of another. For example, donor 1 had a value of 12 pmol/ 10^7 cells, whereas donor 2 had a value of 42. To examine the source of these differences, lymphocytes from donor 4 were examined at biweekly intervals for 2 months to see if his values changed during this period. Table 2(b) shows that his values for monohexosylceramide and lactosylceramide were quite constant, whereas his trihexosylceramide, globoside and paragloboside values tended to vary more. It seems that for glycosphingolipids that occur in large amounts, very consistent results can be obtained, but for glycosphingolipids occurring in very small amounts, it is much more difficult to obtain precisely reproducible data. This may be due to both actual changes in the contents of these minor glycosphingolipids and to difficulties in precisely quantifying glycosphingolipids that occur only at the lower limits of detection. It can be seen in Fig. 1 that peaks corresponding to 6, 9 and 11 are quite small and considerable error can be introduced, even at the level of integration.

Identification of each of these peaks is on the basis of their retention times, because chemical analysis of

such small amounts of material would be impossible. An authentic sample of each of these glycosphingolipids co-chromatographs exactly with the numbered peaks in Fig. 1 and no other known glycosphingolipids chromatograph at the positions of these peaks.

Table 3 shows the results of the analysis of separated T- and B-lymphocytes from the same group of donors as in Table 2. Again, similar variations are seen between donors, mostly in the minor glycosphingolipids. Within the populations of separated T- and B-cells, similar ratios of one glycosphingolipid to another exist. Of significance, though, is that B-cells contain as much as 12 times more of each glycosphingolipid than do T-cells. One obvious explanation for this is that B-cells have a much higher density per cell of these neutral glycosphingolipids, although what functional significance this may have is not known. A second possibility is that the majority of the T-cell glycosphingolipids is expressed only in a minor subpopulation. Preliminary evidence with antibodies to glycosphingolipids in immunofluorescence studies seems to indicate that some T-cell glycosphingolipids are indeed expressed only in about 10% of these cells (Schwartz & Marcus, 1979). H.p.l.c. analysis of the glycosphingolipid content of functional subpopulations of human T- and B-lymphocytes may lead to some insight into the role played by these cell-surface molecules in the development of the immune response.

To further demonstrate the potential of this study, lymphocytes from two patients with chronic lymphatic leukaemia were examined. The majority of these patients' cells are B-lymphocytes, which are thought to be arrested at some point early on in maturation (Klein *et al.*, 1970; Wilson & Nossal, 1971; Preud'homme & Seligman, 1972; Aisenberg & Bloch, 1972). As has been reported previously (Hildebrand *et al.*, 1975), monohexosylceramide is the major glycosphingolipid in these cells, and occurs at near normal concentrations. We have also been able, by the use of this new h.p.l.c. method, to detect the other four glycosphingolipids (lactosylceramide, trihexosylceramide, globoside and paragloboside) in the cells of these patients. These four glycosphingolipids were present in concentrations equal to about half of those in normal T-cells (see Table 3) and were drastically decreased compared with those in normal B-cells. Cell transformation is associated with a simplification of the pattern of glycosphingolipids in many systems, and chronic lymphatic-leukaemia cells apparently conform to this generalization, the significance of which is not understood.

At one point, we would have hoped to report values for glycosphingolipids of Epstein-Barr-virus-transformed cultured human B-cell lines, but found

the concentrations of their glycosphingolipids to be totally unrelated to those in normal B-cells or even chronic-lymphatic-leukaemia cells. These concentrations appear to be highly affected by culture conditions. This high-lights the significance of the present paper, in that the sensitivity of this method allows us to examine the chemical constituents of uncultured lymphocytes from individual donors, and should be of value in elucidating the role of these molecules in normal lymphocytes as well as in the diseased state.

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