Neutral glycolipids in leukemic and nonleukemic leukocytes

J. HILDEBRAND, P. STRYCKMANS, and P. STOFFYN*

Department of Internal Medicine and Clinical Investigation,
Institut Jules Bordet, Brussels University, Brussels, Belgium

ABSTRACT Neutral lipids, free and total cholesterol, glycolipids, and phospholipids were determined in 20 preparations of leukocytes distributed in four groups. Group I consisted of leukocytes from nonleukemic patients; group II, from patients with chronic myelogenous leukemia; group III, from patients with chronic lymphocytic leukemia; and group IV, from patients with acute leukemia.

Two neutral glycolipids were found in nonleukemic mixed leukocyte populations. They were identified as glucosylceramide and lactosylceramide. The same glycolipids were also present in leukemic cells, but striking differences in glycolipid composition were found in various types of leukocytes.

Glycolipids accounted for 8.9–12.6% of the total lipids in leukocytes from group I, 11.4–20.4% in group II, 1.2–1.6% in group III, and 0.5–4.9% in group IV.

Glucosylceramide was the only glycolipid found in seven out of eight analyzed samples of lymphocytes, both normal and leukemic. Lactosylceramide was the major glycolipid in preparations consisting mainly of polymorphonuclear, myeloid, and blastic cells. Only lactosylceramide was found in platelets, where its concentration was about 100 times lower than in mixed leukocyte populations.

SUPPLEMENTARY KEY WORDS   glucosylceramide · lactosylceramide

STUDIES of potentially antigenic substances of neoplastic tissues are of great interest, since there is now enough evidence that the host is able to produce antibodies against malignant tumors (1).

Glycolipids are present in various tumors (2, 3), and they have been found to have antigenic properties. Rapport and his associates demonstrated that cytolipin H, a glycolipid of relatively simple chemical structure and low molecular weight, contributed to the antigenicity of certain neoplasms (4, 5). In addition, glycolipids of a peculiar structure containing fucose were found in some carcinomas (6).

Determination of glycolipids present in malignant cells of solid tumors is complicated by the presence of variable amounts of connective tissue, which cannot be easily dissociated from the neoplastic components, and by the low concentration of these lipids in most tumors.

In contrast with solid tumors, glycolipids account for an exceptionally high percentage of total lipids in populations of mixed leukocytes (7). Moreover, substantial amounts of almost pure leukocytes may be isolated from the peripheral blood of leukemic and nonleukemic patients.

Comparative studies of lipids in leukocytes from patients with various types of leukemia have been previously performed (8), but glycolipids were not investigated.

The purpose of this study was to determine the qualitative and quantitative composition of glycolipids in various forms of leukemic and nonleukemic leukocytes in order to detect possible differences.

MATERIALS AND METHODS

20 preparations of leukocytes were used for the determination of neutral lipids, free and total cholesterol, glycolipids, and phospholipids. They were distributed in four groups. Preparations of group I, used as nonleukemic controls, were from patients with solid tumors. This group also included one preparation of pooled leukocytes from normal donors. Group II was formed

by four patients with chronic myelogenous leukemia (CML), group III by four patients with chronic lymphocytic leukemia (CLL), and group IV by six patients with acute leukemia, both myeloblastic (AML) and lymphoblastic (ALL).

In addition, determinations of glucosylceramide and lactosylceramide were performed in four preparations of erythrocytes, four of lymphocytes (obtained by catheterization of the lymph duct), and two of platelets. All these preparations were from nonleukemic patients.

Isolation of Leukocytes

Leukocyte-enriched fractions were obtained from the whole blood by an IBM experimental blood-cell separator or were collected after sedimentation in a plastic bag containing an anticoagulant solution of ACD formula A (75 ml of 0.8% citric acid, 2.2% sodium citrate, and 2.45% dextrose). Further purification of leukocytes was achieved as follows. After addition of 0.15 to 0.20 vol of Plasmagel solution (Laboratoire Roger Bellon, Neuilly, France), the preparations were kept at 40°C for 25–40 min to allow erythrocytes to sediment. The supernatant was then centrifuged at 800–1000 rpm to remove the platelet-rich plasma. The leukocyte pellet was washed three times with 0.88% NaCl solution and, after each washing, the erythrocytes, which sedimented to the bottom of the centrifugation tube, were discarded. Final preparations contained 2–15 erythrocytes and 15–40 platelets per 100 leukocytes except in two cases, 8 and 10, where the count of platelets was 220 and 240 per 100 leukocytes.

Platelets that were isolated by the IBM separator, erythrocytes collected after sedimentation of the whole blood, and lymphocytes from the lymph duct were practically free of other blood elements.

Extraction and Separation of Lipids

Total lipids were first extracted with 20 vol of chloroform–methanol 2:1 (v/v) for 12–16 hr with occasional shaking and then with 5 vol of chloroform–methanol 1:1 (v/v) for 2 hr. The two lipid extracts were then combined, and enough chloroform was added to bring the final ratio of chloroform–methanol to 2:1 (v/v). The partition into two phases was achieved by addition of 0.2 vol of 0.88% KCl, according to the method of Folch, Lees, and Sloane Stanley (9). The lower phase was evaporated under vacuum and transferred to a Florisil column (Floridin Co., Tallahassee, Fla.). Lipids were eluted stepwise according to the following schema: (a) pure chloroform, (b) chloroform–methanol 95:5 (v/v), (c) chloroform–methanol 2:1 (v/v), and (d) water-saturated chloroform–methanol 2:1 (v/v). After elution from the Florisil column, glycolipids of fractions (b) and (c) were submitted to mild alkaline hydrolysis (10).

Identification of Glycolipids

Glycolipids, resistant to mild alkaline hydrolysis, were characterized by cochromatography on TLC plates with authentic standards and using anthrone–H_2SO_4 as spray reagent, by identification of the sugars, and by determination of the molar ratios between different sugars and between sphingosine and hexoses.

TLC was performed on Silica Gel G or Silica Gel H plates with the following solvent systems: Silica Gel G, chloroform–methanol–water 65:25:4 and 65:35:4 (v/v/v), and n-propanol–water 7:3 (v/v); Silica Gel H, tetrahydroxyfurane–methanol–methylal-2 N NH_4OH 50:25:25:1 (v/v/v/v), chloroform–methanol–concentrated ammonia 6:4:1 (v/v/v), and n-propanol–water–concentrated ammonia 80:13.7:6.7 (v/v/v). In addition, monohexosylceramides were chromatographed on borate-treated Silica Gel G plates in chloroform–methanol–water 65:25:4 (v/v/v). This system, devised by Young and Kanfer, allows the separation of glucosylceramide and galactosylceramide (11).

After acid methanolysis of the glycolipids, the methanolyzate was put on a small column containing charcoal–Celite 535, and the hexosides were eluted with 80% methanol. Acid hydrolysis of the hexosides with 2 N H_2SO_4 yielded the free sugars, which were separated by descending chromatography on Whatman paper No. 3MM developed in ethyl acetate–pyridine–water 12:5:4 (v/v/v) (12).

Analytical Methods

Proteins were determined by the method of Lowry, Rosebrough, Farr, and Randall (13), using bovine serum albumin as a standard. Neutral lipids were determined by the procedure of Zöllner and Kirsch (14), cholesterol was measured according to the method of Sperry and Webb (15), and phosphorus was estimated by the procedure of Fiske and SubbaRow (16). Phospholipids were calculated by multiplying the phosphorus values by 25. Glycolipids were determined by a modification of the method of Greaney (17). 8–30 μg of standards, glucosylceramide or cytolipin H, and samples of isolated glycolipids were dried in 12-ml tubes and placed in boiling water for 15 min after addition of 0.5 ml of acid–orcinol reagent (5 mg of orcinol per ml of 53% H_2SO_4). After hydrolysis the tubes were cooled in ice and 6 ml of chloroform–methanol 1:1 (v/v) was added to each sample. The absorbance at 440 nm was measured on a Beckmann spectrophotometer against an acid blank.

Two procedures were followed to establish the molar ratio between glucose and galactose. In the first proce-

dure the total hexoses were determined by the orcinol method, and glucose was determined with glucose oxidase, using Glucostat reagent (Worthington Biochemical Corp., Freehold, N.J.). In the second procedure, glucose and galactose were determined with anthrone (18) after separation of the sugars by paper chromatography and their elution from the paper as described by Spiro (19).

Sphingosine was determined according to Schmidt et al. (20).

Reagents

Glucosylceramide was prepared from beef kidney, galactosylceramide was from human white matter, and globoside was isolated from human erythrocytes following the procedure described by Mårtensson (21) for isolation of neutral glycolipids from human kidney. Lactosylceramide (cytolipin H) was obtained from Miles Laboratories, Inc. (Elkhart, Ind.).

RESULTS

Leukocyte Composition of the Preparations

The distribution of various types of leukocytes in the analyzed preparations is given in Table 1. In cases 3, 5, and 18, the poor preservation of the cells after the isolation procedures did not allow a proper morphologi-

cal examination. In these cases, differential counts of leukocytes were established on peripheral blood samples taken prior to the isolation.

Leukocytes isolated from nonleukemic patients (group I) are a mixture of white cells normally found in peripheral blood. In this group PMN are predominant, and they account for 68–87% of the white cells; lymphocytes and monocytes contribute only 12–32%. In group II (CML) 88–100% of cells belong to the myeloid series. In group III (CLL) almost all cells are small lymphocytes. Contrasting with the other groups, marked individual differences in cell-type composition are found in group IV. In case 15 (AML) myelocytes and monocytes account for half of the analyzed cells. The five other preparations contained, in various proportions, blastic cells and lymphocytes.

Lipid Determination

Table 2 shows the lipid composition of the leukocytes. Lymphocytes isolated from patients with CLL (group III) had the highest lipid concentration. With the exception of the nonleukemic patients, cholesterol appeared as the major neutral lipid. In all groups the ratio of free to total cholesterol was high, averaging 85.1%.

Phospholipids were the main fraction in all preparations. Their levels were particularly high in lymphocytes (group III). However, the most striking differences, both qualitative and quantitative, between the analyzed

TABLE 1   DIFFERENTIAL LEUKOCYTE COUNT OF THE ANALYZED PREPARATIONS

Group	Case Number	Diagnosis	PMN Neutrophilic	PMN Basophilic	PMN Eosinophilic	Meta-myelocytes	Myelocytes	Pro-myelocytes	Blastic Cells	Lymphocytes	Monocytes
						% of total leukocytes					
I	1	NL*	73		1					21	5
	2	NL	69.5		3					18.5	9
	3†	NL	86		1					6	7
	4	NL	83		1					14	2
	5†	NL	87		1					10	2
	6	PNL	68							28	4
II	7	CML	14	6		16	44	8	6	6	
	8	CML	62	1	4	10	19	4			
	9	CML	65		1	12	20	1		1	
	10	CML	51	1	1	24	12	5	3		3
III	11	CLL								100	
	12	CLL	1							98	1
	13	CLL	2							98	
	14	CLL	4		1					93	2
IV	15	AML	6			12	13		37	6	26
	16	AML							98	2	
	17	AML	0.5		1			0.5	75.5	18	4.5
	18†	ALL							80	16	4
	19	ALL	1						98	1	
	20	ALL	0.5						40.5	58	1

Abbreviations: PMN, polymorphonuclear cells; NL, nonleukemic patients; PNL, pooled leukocytes from normal volunteers; CML, chronic myelogenic leukemia; CLL, chronic lymphogenic leukemia; AML, acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia.

* Counted on 200 cells.

† Differential count determined on peripheral blood.

TABLE 2 LIPIDS OF HUMAN LEUKOCYTES

Group	Case Number	Diagnosis	Total Lipids	Neutral Lipids	Total Cholesterol	Free Cholesterol	F/T*	Glucosyl-ceramide	Lactosyl-ceramide	Phospho-lipids
I	1	NL	96.5	43.7	21.4	18.4	85.2	0.4	10.7	41.7
	2	NL	110.4	45.5	21.4	18.3	85.1	0.6	10.4	53.9
	3	NL	105.2	36.2	33.1	31.6	95.4	0.2	9.5	59.2
	4	NL	115.8	42.5	ND†	ND	ND	0.2	12.4	60.7
	5	NL	128.5	56.2	ND	19.2	ND	0.5	15.7	56.1
	6	PNL	119.7	50.5	32.5	23.4	72.0	TR‡	10.8	58.4
II	7	CML	95.3	36.2	18.6	ND	ND	0.8	10.1	48.2
	8	CML	105.5	26.0	20.6	19.2	93.3	0.8	15.6	63.1
	9	CML	102.5	21.7	20.7	19.1	92.6	1.2	11.5	68.1
	10	CML	85.9	20.4	19.3	17.7	91.7	0.9	16.5	48.1
III	11	CLL	146.4	25.4	18.4	17.1	92.2	2.1	0	118.9
	12	CLL	ND	19.4	18.1	14.7	81.4	1.2	0	ND
	13	CLL	137.4	28.2	23.0	21.5	93.1	2.2	0	107.0
	14	CLL	155.5	33.2	25.0	22.1	88.4	1.8	0	120.5
IV	15	AML	134.5	28.9	12.5	11.4	91.2	0.2	5.8	99.6
	16	AML	100.6	24.8	22.3	21.1	94.6	TR	0.5	75.3
	17	AML	90.9	19.8	20.8	17.0	81.2	0.9	1.4	68.3
	18	ALL	85.4	21.0	19.7	5.8	29.5	1.3	2.9	60.2
	19	ALL	135.0	37.4	37.2	35.6	95.3	TR	4.4	93.2
	20	ALL	95.0	24.6	14.6	12.7	86.9	1.0	0.6	68.8

The results are expressed in mg of lipids per g of protein. Abbreviations are the same as in Table 1.
* F/T, ratio of free to total cholesterol.
† ND, not determined.
‡ TR, traces.

groups of leukocytes were found in the glycolipid fraction.

Identification of Glycolipids in Human Leukocytes

Two glycolipids were isolated from leukocytes of group I, and they were identified as glucosylceramide (CMH) and lactosylceramide (CDH).

The glycolipid identified as CMH, eluted from a Florisil column with chloroform–methanol 95:5 (v/v), migrated like authentic glucosylceramide standard on TLC in six different solvent systems. In addition, on borate-treated Silica Gel G plates, the R_F value of this lipid was identical to that of glucosylceramide and greater than that of galactosylceramide (cerebroside). After acid hydrolysis, the carbohydrate moiety was identified as glucose. The molar ratio of glucose to sphingosine was 0.91.

The second glycolipid, characterized as CDH, was eluted from a Florisil column with chloroform–methanol 2:1 (v/v). It migrated on TLC to the same position as authentic lactosylceramide (cytolipin H). Its carbohydrate moiety consisted of galactose and glucose in the molar ratio of 1.09 when the sugar determination was made by the orcinol and glucose oxidase methods, and 1.21 when hexoses were measured with anthrone. The values of the ratio in CDH extracted from leukocytes of groups II and IV were, respectively, 1.08 and 0.99 by orcinol and glucose oxidase procedures, and 1.37 and 1.22 by the anthrone procedure. The molar ratio of hexose to sphingosine was 2.11.

A third anthrone-positive lipid was eluted from the Florisil columns with water-saturated chloroform–methanol 2:1 (v/v); this lipid was mainly from samples with the highest erythrocyte contamination. It migrated on TLC like globoside (tetrahexosylceramide).

Determination of CMH and CDH in Erythrocytes and Platelets

CMH and CDH determinations were also performed in erythrocytes and platelets, since the analyzed leukocyte preparations contain these elements. The concentration of CDH in human erythrocytes ranged from 42 to 65 μg and in platelets from 80 to 100 μg per g of protein.

Distribution of Glycolipids in Various Forms of Leukocytes

The percentage of glycolipids, both CMH and CDH, as compared with the total lipids, varied considerably from one group of leukocytes to another (Fig. 1).

In mixed leukocyte populations, isolated from non-leukemic patients (group I), both CMH and CDH were present. The concentration of the two glycolipids ranged from 9.7 to 16.2 mg per g of protein, and they represented 8.9–12.6% of the total lipids. CDH was the major glycolipid and accounted for 97% of the total glycolipids.

In group II (patients with CML), the levels of the glycolipids ranged from 10.9 to 17.4 mg/g protein. Glycolipids made up 11.4–20.4% of the total lipids. Again CDH was the major glycolipid, but the concentra-
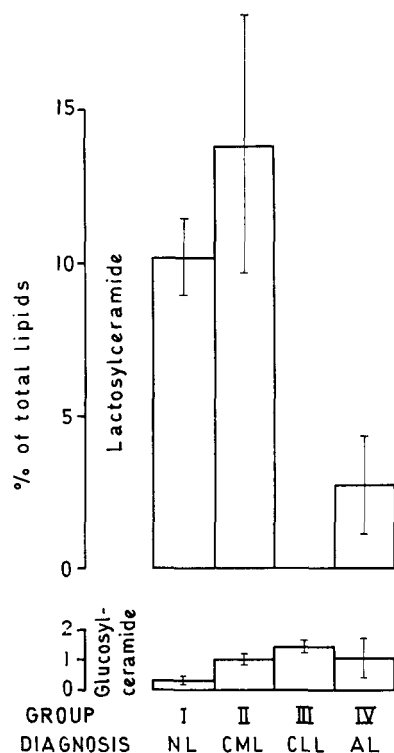
FIG. 1. Glucosylceramide (CMH) and lactosylceramide (CDH) are expressed as percentages of the total lipids. Mean values and standard deviations are given for each group.

tions of CMH were significantly higher than in group I ($P < 0.01$).

In lymphocytes isolated from patients with CLL (group III), the amounts for total glycolipids were considerably lower than in the two previous groups. They ranged from 1.2 to 2.2 mg per g of protein, which represents 1.2–1.6% of the total lipids. The only glycolipid found in this group was CMH.

In lymphocytes obtained by catheterization of the lymph duct of normal donors, CMH was the only glycolipid in three preparations; its concentration ranged from 0.72 to 0.98 mg per g of protein. In a fourth sample, however, only CDH was found in slightly higher concentration, 1.40 mg per g of protein.

Both CMH and CDH were found in leukocytes isolated from patients with acute leukemia (group IV). However, appreciable amounts of CMH were present only in preparations which contained lymphocytes (cases 17, 18, and 20). The concentrations of CDH ranged from 0.5 to 5.8 mg per g of protein. Total glycolipid levels ranged from 0.5 to 6.0 mg and they accounted for 0.5–4.9% of the total lipids.

DISCUSSION

Two glycolipids are found in mixed leukocyte populations isolated from normal volunteers and patients with solid tumors. The major glycolipid, which accounts for more than 95% of total glycolipids, is a dihexosylceramide containing glucose and galactose. Since the ratio of the two hexoses is close to 1, it is concluded that this lipid is mainly, if not exclusively, a lactosylceramide (CDH). This result does not agree with the data presented by Miras, Mantzos, and Levis (7). According to these authors, the dihexosylceramide is a mixture of lactosylceramide and digalactosylceramide. We have no explanation for this discrepancy.

The second glycolipid isolated from the nonleukemic leukocytes is glucosylceramide (CMH).

Traces of a third anthrone-positive lipid are found in the samples with the highest erythrocyte contamination. This lipid has the same R_F values as the globoside which is known to occur in considerable concentration in human erythrocytes (22). Its presence in the lipid extracts may be due to the contamination of the leukocyte preparations by red cells.

The sum of CMH and CDH accounted for 8.9–12.4% (mean 10.3) of the total lipids. These figures are considerably lower than the values reported by Miras et al. (7) (15.2 and 16.4%). The difference may be partially explained by a somewhat higher contamination of our preparations by platelets and erythrocytes. Indeed, the concentration of CDH in red cells and platelets is more than 100 times lower than in mixed leukocyte preparations.

However, despite the previously mentioned differences, our data confirm the exceptionally high concentration in leukocytes of dihexosylceramide previously observed by Miras et al. (7).

The same glycolipids are present in leukemic and in nonleukemic leukocytes, but marked variations in the composition and concentration of those lipids were found in different types of leukocytes.

High levels of glycolipids were found in leukocytes from groups I and II, which mainly consisted of PMN and myeloid cells. In contrast, the concentrations of glycolipids in lymphocytes are considerably lower, and the values are similar to those of other nonnervous tissues. In addition, though CDH is the major glycolipid in myeloid series cells, only CMH was found in all lymphocyte preparations from patients with CLL and in three out of four samples of normal lymphocytes. However, in the fourth preparation of normal lymphocytes, CDH was present. Whether this difference is due to individual variation or whether the examined lymphocytes belong to different populations is now under investigation.

In patients with acute leukemia, appreciable amounts of CMH were found in preparations containing lymphocytes, but in preparations that consisted almost exclusively of blastic cells (cases 16 and 19), CMH was

present only in trace amounts. This suggests that, as in groups I and II, CDH is the main glycolipid in blastic cells. However, the concentrations of this glycolipid are significantly lower in blastic cells than in leukocytes from the first two groups, although the levels of other lipids are similar in groups I, II, and IV. The pattern of glycolipids, mainly gangliosides, may be different in normal and neoplastic cells (23, 24); however, if we assume that the amounts of glycolipids present in leukemic and nonleukemic blastic cells are similar, our data indicate a striking increase of CDH in PMN cells during the maturation processes. Such an increase of CDH concentrations might be related to some peculiar function of the PMN cells. The role and the subcellular distribution of CDH in PMN leukocytes is now under investigation.

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