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RAPID DETERMINATION OF LIPIDS IN HEALTHY HUMAN LYMPHOCYTES

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SUMMARY

Lipids of human lymphocytes were determined from 20 ml of heparinized peripheral blood using thin-layer chromatography with a flame ionization detector, and gas chromatography.

The weight per cent and μg per 10^6 lymphocytes for cholesterol ester, triglyceride, free cholesterol and phospholipid were 11.1 and 5.2, 18.1 and 17.9, 15.1 and 8.5, and 55.7 and 44.2, respectively. Phospholipid was the major lipid component in human lymphocytes. Phospholipid was subfractionated into phosphatidylethanolamine, phosphatidylinositol plus phosphatidylserine, phosphatidylcholine and sphingomyelin in amounts of 25.2, 6.1, 46.9 and 22.9%, respectively.

Total fatty acid composition was analyzed as: $C_{14:0}$, 13.3%; $C_{16:0}$, 20.9%; $C_{16:1}$, 6.5%; $C_{18:0}$, 19.6%; $C_{18:1}$, 18.8%: $C_{18:2}$, 7.1%; and $C_{20:4}$, 12.3%. Higher cholesterol ester and triglyceride and lower $C_{14:0}$ were characteristic of female lymphocytes when compared with male lymphocytes.

The lipid composition quantitated by this method corresponded well with previously reported data.

Thus, this method can be used clinically because of its simplicity and higher sensitivity.

INTRODUCTION

Human B lymphocytes possess a low density lipoprotein receptor [1] which controls intracellular cholesterol metabolism by regulating 3-hydroxy-3-methylglutaryl coenzyme A reductase activity [2, 3]. Lipids in lymphocytes have to be determined rapidly and with higher sensitivity because of the low density lipoprotein receptor activity. However, there have been very few reports describing lipid analysis in human lymphocytes because of the difficulties and unreliability of obtaining adequate amounts of suitably pure lymphocytes for analysis. Gottfried [4] has already described the lipid composition of leuko-

cytes as well as of lymphocytes which were separated from 250 ml of peripheral blood.

A microanalytical technique starting with 20 ml of peripheral blood using a newly developed method of thin-layer chromatography (TLC) connected to a flame ionization detector and gas chromatography is described in this paper.

MATERIALS AND METHODS

Subjects

Five females and six males, for whom the physical and laboratory examinations were determined to be within normal limits, were examined. Their mean age, serum total cholesterol, triglyceride and phospholipid were 25 ± 2 years, 162 ± 25 mg/dl, 75 ± 6 mg/dl and 169 ± 18 mg/ml, respectively.

Purification of lymphocytes

A 20-ml sample of heparinized venous blood was drawn from each subject after overnight fasting. Silica solution (KAC II, Nippon Kotai Lab., Takasaki, Japan) was added at a concentration of 1/10 volume and incubated for 30 min at 37°C for depletion of monocytes from the mononuclear cell population. Then the lymphocytes were separated by Böyum's method [5]. The cells were washed twice with phosphate-buffered saline (pH 7.2) containing 2% bovine serum albumin (Sigma, St. Louis, MO, U.S.A.) by centrifugation for 10 min at 180 *g* to diminish platelet contamination.

The lymphocytes were washed twice with 0.15 *M* NaCl solution containing 1 mg/ml EDTA (Wako Chem., Osaka, Japan) and the number of lymphocytes was calculated using a Unopet (Becton-Dickinson, Rutherford, NJ, U.S.A.).

The purity of the lymphocytes was determined by May—Grünwald—Giemsa staining. The number of cells other than the lymphocytes in the purified lymphocyte sample was 10—20 platelets, fewer than 5 neutrophils, and no red blood cells per 100 lymphocytes. The total number of lymphocytes obtained from 20 ml of peripheral blood was approximately $1.5 \cdot 10^7$—$2.0 \cdot 10^7$. The sample tube was filled with nitrogen and stored at −70°C until the lipid analysis.

Extraction of lipids from lymphocytes

Lipids were extracted from lymphocytes with a mixture of chloroform—methanol (2:1, v/v) containing cholesterol acetate at a concentration of 10 mg/dl as an internal standard. After the solvents had been rinsed twice with saline, the top layer was discarded and the lower and intermediate layers were filtered through filter paper Type 5A (Toyo Co., Tokyo, Japan). The crude lipid extracts were dried in a rotary evaporator at a temperature below 37°C, and the lipid was redissolved immediately in 200 μl of cold chloroform—methanol (2:1, v/v).

Of the 200 μl, 9 μl were used for analysis of neutral lipids and 40 μl for fatty acid composition. The remaining 151 μl were evaporated again, redissolved in 10 μl of cold chloroform—methanol (2:1, v/v) and used for analysis of phospholipid subfractions.

Thin-layer chromatography combined with flame ionization detection

TLC was performed on a Chromarod (Iatron, Tokyo, Japan), which was a quartz rod, 0.9 mm in diameter and 152 mm long, coated with silica gel. After chromatography the rod was passed through a flame ionization detector (Iatron) under the following conditions: hydrogen pressure 1.4 kg/cm^2, air flow-rate 2000—2500 ml/min, scanning speed gear 40T, chart speed 240 mm/min, and recorder 0.1 V.

Each fraction detected by the flame ionization detector, was identified using a purified standard, such as cholesterol ester, triglyceride, free fatty acid and phosphatidylcholine (Sigma), and the values were quantitated by measuring the step height of the integrating signal in triplicate samples. The height ratio of individual neutral lipids to the internal standard (cholesterol acetate) was divided by the sum of the ratio and expressed as weight per cent. For the cholesterol, cholesterol ester, triglyceride and phosphatidylcholine, calibration curves were obtained by adding various amounts of each standard lipid and the values of each lipid in the lymphocytes were expressed as μg per 10^6 lymphocytes.

Lipid phosphorus of five lymphocyte samples was determined with the Fiske—SubbaRow reagent [6] in parallel with TLC with flame ionization detection.

Analysis of neutral lipids

Of the 9 μl of lipid extract 3 μl were applied to each of three Chromarods for TLC. Total lipids on the Chromarods were moved in the first phase 10 mm from the point of application and then air-dried. Neutral lipids, consisting of cholesterol ester, cholesterol acetate, triglyceride, free cholesterol and phospholipid, were developed in a mixture of *n*-hexane—diethyl ether (9:1, v/v) for 40 min at 20°C.

The Chromarod was passed through a flame ionization detector after drying for 3 min at 110°C. The values of neutral lipids in the lymphocytes were expressed as the mean of the three samples.

Analysis of phospholipids

The procedures for analysis of phospholipids were the same as those described above. Phospholipid was subfractionated in chloroform—methanol—water (60:30:3.5, v/v), containing 500 mg/dl 2,4-di-*tert.*-butylphenol (BHT). Each fraction was identified using a standard mixture of phosphatidylcholine, sphingomyelin (Sigma), phosphatidylethanolamine (purified from *Escherichia coli*), phosphatidylinositol (purified from yeast) and phosphatidylserine (purified from bovine brain) (by courtesy of Prof. S. Nozima, School of Pharmacy, Tokyo University, Tokyo, Japan).

Each fraction was expressed as a percentage by measuring the step height of the integrating curve of each fraction and calculating the weight per cent compared with that of total phospholipid.

Analysis of fatty acids

Forty microliters obtained from 200 μl of lipid extract were evaporated again to dryness. Dried lipid was methylated using boron trifluoride com-

pounds (Wako). Methylated fatty acids were analyzed by gas chromatography (Model 163P, Hitachi Co., Tokyo, Japan). A glass column 2 m × 3 mm, was packed with 5% Chromosorb W AM DMCS (80—100 mesh treated with free fatty acid polyester as a liquid phase (Chromato Res. Co., Sagamihara, Japan). The injection temperature of the sample was 230°C, the column temperature was 200°C, nitrogen gas flowed at 300 ml/min and hydrogen at 30 ml/min with air at 500 ml/min.

Statistical evaluation

Student's *t*-test was employed for statistical evaluation.

RESULTS

Chromatographic patterns of neutral lipids and phospholipid subfraction

Cholesterol ester, triglyceride, free fatty acid, free cholesterol and phospholipid were determined as neutral lipids of lymphocytes. The peaks of phospholipid, free cholesterol, triglyceride and cholesterol ester were sharp and high, although the peak of free fatty acid was lower and, in many cases, unrecognizable (Fig. 1).

The peak of cholesterol acetate as an internal standard did not overlap any peak of the other lipids. Phospholipids were subfractionated into four peaks corresponding to phosphatidylethanolamine, phosphatidylinositol plus phosphatidylserine, phosphatidylcholine and sphingomyelin (Fig. 2). Phosphatidylinositol and phosphatidylserine were rarely separated into two fractions by this method.

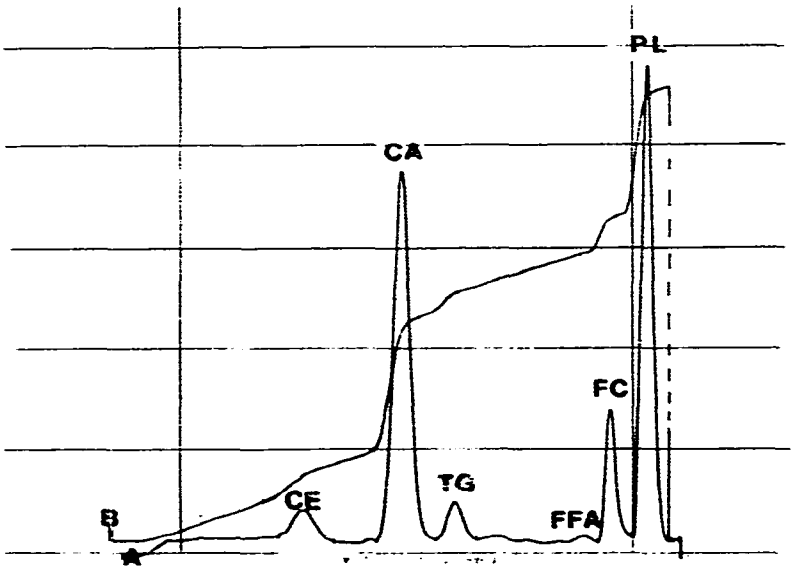

Fig. 1. Pattern of neutral lipids and phospholipids of whole lymphocytes obtained by TLC with flame ionization detection. A = differential curve, B = integrating curve, CE = cholesterol ester; CA = cholesterol acetate, TG = triglyceride, FFA = free fatty acids; FC = free cholesterol, and PL = phospholipids.

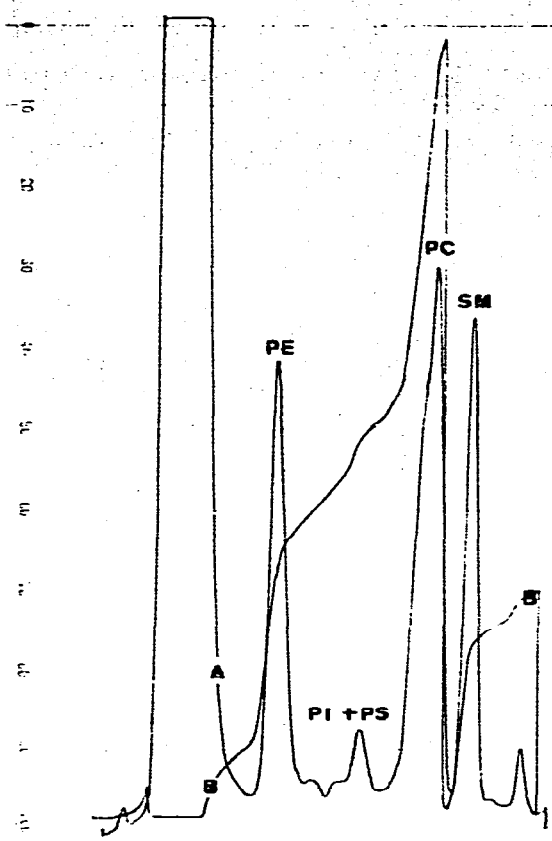

Fig. 2. Pattern of phospholipid subfractions obtained by TLC with flame ionization detection. A = Differential curve, B = integrating curve, PE = phosphatidylethanolamine, PI + PS = phosphatidylinositol plus phosphatidylserine, PC = phosphatidylcholine, SM = sphingomyelin.

Quantitation of neutral lipids and phospholipids

Phospholipids (55.7% of the lipid extract) were the major lipid component in the lymphocytes, i.e. 44.2 μg per 10^6 lymphocytes, and there was no statistical difference between males and females. Triglyceride (18.1% of the lipid extract, and 17.9 μg per 10^6 lymphocytes) was another major component of the lipids.

Triglyceride in the female lymphocytes was higher than in the male lymphocytes ($P < 0.001$). Cholesterol ester and free cholesterol were nearly equal in the lipids of the lymphocytes. Cholesterol ester in the female lymphocytes was also higher than in male lymphocytes ($P < 0.01$) (Table I).

The molar ratio of free cholesterol to phospholipid was 0.56, and no sex difference existed.

Phospholipid in 10^6 lymphocytes was 42.1 ± 5.4 μg by TLC and 37.5 ± 4.2 μg by colorimetry using Fiske—SubbaRow reagent, and a good correlation coefficient (0.96, $P < 0.01$) was obtained (Table II).

Quantitation of phospholipid subfractions

Phosphatidylcholine (46.9%) was the major component in the subfractions

TABLE I

LIPID COMPOSITION AND THE MOLAR RATIO OF FREE CHOLESTEROL TO PHOSPHOLIPIDS OF MALE AND FEMALE LYMPHOCYTES

Lipids were analysed by TLC with flame ionization detection and expressed as weight per cent and μg per 10^6 lymphocytes. Figures in parentheses are the number of experiments. Values are expressed as mean ± S.D.

		Total (11)	Female (5)	Male (6)
Cholesterol ester	(weight per cent)	11.1 ± 3.8	10.1 ± 3.1	11.1 ± 4.5
	(μg per 10^6 cells)	5.2 ± 2.0	7.5 ± 1.5*	4.5 ± 1.2
Triglyceride	(weight per cent)	18.1 ± 9.7	22.9 ± 6.2	14.2 ± 10.9
	(μg per 10^6 cells)	17.9 ± 12.9	27.3 ± 8.5**	5.4 ± 1.1
Free cholesterol	(weight per cent)	15.1 ± 4.8	12.7 ± 3.7	17.0 ± 5.0
	(μg per 10^6 cells)	8.5 ± 1.9	9.5 ± 0.1	7.8 ± 1.2
Phospholipid	(weight per cent)	55.7 ± 7.9	54.0 ± 5.7	57.0 ± 9.7
	(μg per 10^6 cells)	44.2 ± 12.9	50.5 ± 18.1	39.1 ± 8.1
Molar ratio of free cholesterol/phospholipid		0.56 ± 0.11	0.51 ± 0.11	0.59 ± 0.09

*$P < 0.01$.
**$P < 0.001$.

TABLE II

COMPARISON OF PHOSPHOLIPIDS DETERMINED BY TLC WITH FLAME IONIZATION DETECTION AND THE COLORIMETRIC METHOD

Values are expressed as μg per 10^6 lymphocytes. Correlation coefficient is 0.96 ($P < 0.01$).

Sample No.	TLC with flame ionization detection	Colorimetric method
1	37.7	32.8
2	38.5	34.9
3	40.1	37.2
4	43.1	39.1
5	51.5	43.7
Mean ± S.D.	42.1 ± 5.4	37.5 ± 4.2

TABLE III

PHOSPHOLIPID DISTRIBUTION IN MALE AND FEMALE LYMPHOCYTES

The phospholipid distribution was analyzed by TLC with flame ionization detection and expressed as weight per cent. Figures in parentheses are the number of experiments. Values are expressed as mean ± S.D.

	Total (11)	Female (5)	Male (6)
Phosphatidylethanolamine	25.2 ± 2.5	23.4 ± 2.9	22.5 ± 2.3
Phosphatidylinositol plus phosphatidylserine	6.1 ± 2.2	6.3 ± 2.5	5.9 ± 2.2
Phosphatidylcholine	46.9 ± 2.6	47.1 ± 3.1	46.8 ± 2.4
Sphingomyelin	22.9 ± 2.5	23.4 ± 2.9	22.5 ± 2.3

of phospholipid. Phosphatidylethanolamine (25.2%) and sphingomyelin (22.9%) followed phosphatidylcholine. Phosphatidylinositol and phosphatidylserine combined (6.1%) was the smallest component (Table III). No sex difference was noted in any of these phospholipid subfractions.

Quantitation of free fatty acids

$C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ were determined as approximately 20% of total free fatty acid using gas chromatography. $C_{14:0}$ was 13.3% and $C_{20:4}$ was 12.3%. $C_{16:1}$ and $C_{18:2}$ were minor components. $C_{14:0}$ in male lymphocytes was higher than in female lymphocytes ($P < 0.05$) (Table IV).

TABLE IV

FATTY ACID COMPOSITION OF MALE AND FEMALE LYMPHOCYTES

Fatty acid composition was analysed by gas chromatography and expressed as weight per cent. Figures in parentheses are the number of experiments. Values are expressed as mean ± S.D.

	Total (11)	Female (5)	Male (6)
$C_{14:0}$	13.3 ± 5.3	10.3 ± 3.6*	17.1 ± 4.7
$C_{16:0}$	20.9 ± 3.9	21.6 ± 3.2	20.1 ± 4.9
$C_{16:1}$	6.5 ± 1.9	6.1 ± 1.9	6.9 ± 2.1
$C_{18:0}$	19.6 ± 2.5	20.7 ± 2.9	18.4 ± 1.0
$C_{18:1}$	18.8 ± 3.5	19.2 ± 4.5	18.5 ± 2.5
$C_{18:2}$	7.1 ± 1.8	7.9 ± 1.9	6.3 ± 1.5
$C_{20:4}$	12.3 ± 4.2	12.2 ± 5.8	12.4 ± 3.1

*$P < 0.05$.

Total lipids

Total lipids extracted from $1.5 \cdot 10^7$—$2.0 \cdot 10^7$ lymphocytes were calculated as approximately 1.0—1.5 mg by quantitation of cholesterol ester, triglyceride, free cholesterol and phospholipid.

DISCUSSION

The molar ratio of free cholesterol to phospholipids has recently been considered as an important factor in regulating membrane fluidity [7]. Recently, experimental approaches to determine the molar ratio of cholesterol to phospholipids from the plasma membrane of lymphocytes were undertaken using various techniques [8]. However, these investigations are hardly applicable to clinical use because of difficulties in obtaining sufficient pure membrane preparations from patients. An improved lipid analysis of the whole lymphocytes is necessary for clinical applications [4]. We have developed a new method for lipid analysis of human platelets using TLC coupled with flame ionization detection [9]. This method is quite useful for clinical examination because of its simplicity and higher sensitivity.

In the present study, the method was applied to analysis of lipid composition of whole lymphocytes obtained from healthy males and females. The lymphocytes were prepared from 20 ml of peripheral blood which is approxi-

mately 1/12 of the volume reported previously (250 ml) [4]. In this method, gradient centrifugation of heparinized blood mixed with Ficoll—Conray was used to obtain the lymphocytes instead of a glass bead column [4]; this procedure eliminated hypotonic lysis of red blood cells because no erythrocytes contaminated our preparations. Thus, this procedure to separate the lymphocytes is simple and suitable for treating many samples in a short time.

Total lipids extracted from $1.5 \cdot 10^7$—$2.0 \cdot 10^7$ lymphocytes were calculated as 1.0—1.5 mg, which was approximately ten times more than the 0.1—0.05 mg described previously [4]. However, our lymphocytes contained 10—20 platelets per 100 lymphocytes. Lipids in 10^8 platelets consisted of 7 μg of free cholesterol and 46 μg of phospholipid [8]; therefore, the lipid content of 10—20 platelets, 0.007 μg of free cholesterol and 0.046 μg of phospholipid, is negligible in comparison with 50—100 μg of lipids extracted from 10^6 lymphocytes.

The phospholipids determined by TLC with flame ionization detection were correlated with the phosphorus content in the lipids detected by colorimetry with Fiske—SubbaRow reagent [6].

The molar ratio of free cholesterol to phospholipids in lymphocytes has been reported as 0.29—0.37 [10] and 0.68 [4], while our value was 0.56. Female lymphocytes had higher triglyceride and cholesterol ester and lower $C_{14:0}$ than male lymphocytes. These sex-related differences in lipids in the whole lymphocytes may depend on sex hormones. Although $C_{14:0}$ (myristic acid) was reported as a small fraction with no mention of a sex difference [11], the 10% in female lymphocytes was significantly lower than the 17% in male lymphocytes in this study. Further studies thus need to be undertaken.

The advantages of our new method to quantitate the lipids of lymphocytes are as follows: (1) various lipids can be determined in a single procedure; (2) the molar ratio of free cholesterol to phospholipid can be determined quickly in one procedure; (3) the Chromarod can be utilized repeatedly; (4) the sample quantity required for Chromarod determination of both neutral lipids and phospholipids is very small, and the remainder of the lipid sample can be used for the analysis of fatty acids by gas chromatography; and (5) 20 ml of heparinized blood are sufficient for this procedure.

One disadvantage of our method was the incomplete separation of phosphatidylinositol from phosphatidylserine. The reason for this depends on the impossibility of performing two-dimensional development on the Chromarod and on the small amounts of phospholipids which were extracted from 20 ml of venous blood. Clear identification of these two phospholipids could be achieved by two-dimensional TLC [12]. Another disadvantage of TLC with an ionization detector is the failure to identify the free fatty acid composition of phospholipid fractions for the same reasons. The free fatty acid of phospholipids can be analysed by TLC and gas chromatography if sufficient sample can be obtained. The lymphocytes were reported to contain about 30% phosphatidylethanolamine, most of which is in the plasmalogen form (α-alkenyl, β-acyl glycerophosphorylethanolamine [13], whereas our study revealed 25% phosphatidylethanolamine.

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