Biochimica et Biophysica Acta, 558 (1979) 282—295

BBA 78583

THE COMPOSITION AND FLUIDITY OF NORMAL AND LEUKAEMIC OR LYMPHOMATOUS LYMPHOCYTE PLASMA MEMBRANES IN MOUSE AND MAN *

S.M. JOHNSON and R. ROBINSON

Division of Immunological Medicine, MRC Clinical Research Centre, Watford Road, Harrow, Middlesex, HAI 3UJ (U.K.)

(Received April 6th, 1979)

Key words: Lipid composition; Fluidity; Leukemia; (Lymphocyte)

Summary

The lymphocyte surface membranes from normal and leukaemic or lymphomatous cells from man and mouse were isolated, characterized, and analyzed both biochemically and by diphenyl hexatriene fluorescence polarization. The cholesterol/phospholipid molar ratio for all the pure lymphocyte plasma membranes was 0.45—0.50, and the fluorescence polarization results showed that values much higher than this were not credible. The lipid composition of all the plasma membranes was remarkably similar, except for the concentration of free fatty acids and glycerides.

The latter two were particularily high in the mouse lymphoma membrane and these, rather than a low cholesterol concentration, were responsible for the increased fluidity of the cells.

The most prominent protein in most of the plasma membrane preparations was actin. This is found only by some authors, and its presence probably depends on the method of lymphocyte disruption.

Introduction

The plasma membranes of unstimulated lymphocytes are relatively easy to isolate in a pure form since these cells contain little endoplasmic reticulum and few mitochondria. Much work has therefore been done on their lipid composition, and in particular on their cholesterol/phospholipid ratios. Some previous

* Supplementary data to this article are deposited with, and can be obtained from: Elsevier/North-Holland Biomedical Press, BBA Data Deposition, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. Reference should be made to BBA/DD/118/78583/558 (1979) 282—295. The supplementary information includes data on the major esterified fatty acids of the lymphocyte plasma membrane preparations.

TABLE I

PLASMA MEMBRANE PREPARATIONS FROM MAN AND MOUSE

n.d., not determined, ALL, acute lymphoblastic leukaemia; CLL, chronic lymphocytic leukaemia.

Reference	Species	Lymphocyte	5'-Nucleotidase purification factor	Cholesterol/ phosphatidyl-choline ratio
1. Demus	Man	Normal tonsil	13	0.69
2. Allan and Crumpton	Man	Thymus	n.d.	0.75
3. Crumpton and Snary	Man	Lymphoblastoid cell line BRI 8	49	1.02
4. Marique and Hildebrand	Man	CLL	29	0.38
5. Pratt et al.	Man	Thymus	8	1.3
		CLL	16	0.2
		Lymphoblastoid cell line LA 85C11	7	0.4
6. Ladoulis et al.	Rat: AKR,	Normal splenic	10—15	0.87—0.19
7. Smith et al.	ACI	Normal thymocyte		0.87—0.98
8. Misra et al.	F344 strains			
9. Dods et al.	Mouse	Ascitic leukaemia	20.0	0.77
10. Warley and Cook	Mouse	ALL	Variable	0.99

work on lymphocytes from man, rat and mouse are summarized in Table I.

By studying the fluorescence depolarization of the membrane-soluble probe 1,6-diphenyl-1,3,5-hexatriene, Inbar and Shinitzky and coworkers [11—14] found that human and mouse leukaemic and lymphoma cells were more fluid than normal lymphocytes, and attributed this to a lower cholesterol/phospholipid ratio in the plasma membranes of the pathological lymphocytes.

Further investigations have not entirely supported their conclusions. For example it has been shown that the probe is not confined to the lymphocyte plasma membrane [15,16] as was previously believed, so plasma membranes have to be isolated for study. The cholesterol/phospholipid ratios obtained for purified normal and abnormal plasma membranes shown in Table I, do not appear to support the initial theory of Inbar and Shinitzky. Johnson and Kramers [17] failed to confirm the fluidity difference between human normal and leukaemic lymphocytes and cell plasma membranes, and attributed the difference found by Inbar and Shinitzky to poor cell purification.

None of the authors in Table I used a modern specific cholesterol assay such as gas chromatography or cholesterol oxidase, and some of the older methods are not specific, particularly those using a chromophore in concentrated sulphuric acid [18]. This investigation was undertaken to measure the cholesterol/phospholipid ratio of the lymphocyte plasma membrane by a specific modern method, and to compare and contrast the plasma membranes from human tonsil and chronic lymphocytic leukaemia lymphocytes and mouse splenic and Gardner lymphoma lymphocytes.

Materials and Methods

Diphenyl hexatriene and preservative free tetrahydrofuran were obtained from the Aldrich Chemical Company, Inc. Diphenyl hexatriene was recrystal-

lized from acetone. Radioisotopes came from the Radiochemical Centre, Amersham, U.K. Phospholipids were purchased from Lipid Products Ltd. and cholesterol and triolein from Sigma Chemical Company.

Cells

Normal human lymphocytes were obtained from enlarged but not inflamed tonsils, freshly removed from children and young adults at operation. The purification and characterisation of the cells is described in Johnson and Kramers [17]. Chronic lymphocytic leukaemic lymphocytes in large numbers were a gift from Professor Galton and were obtained by the plasmaphoresis cell separator. In some cases 20-ml blood samples were obtained from patients with a high lymphocyte blood count. Platelets were removed by defibrination and the lymphocytes purified by Ficoll density gradient separation. Any remaining red cells were removed by Boyle's method of ammonium chloride lysis [19].

Mouse normal lymphocytes were teased out of the spleens of CBA mice. Initially the cell culture medium used was RPMI 1640. After filtering and centrifuging at $200 \times g$ for 10 min, the erythrocytes were removed by lysis at 0°C for 20 s in aqueous 10 mM Tris-HCl, pH 7.5 (20°C), then cells were immediately diluted with an equal volume of twice isotonic L 15. The cells were washed twice, being resuspended in 0.135 M NaCl, which was buffered with 10 mM Tris-HCl, pH 7.5 (20°C). Splenic macrophages were estimated using neutral red, as described for the human tonsils [17]. Any attempt to remove them resulted in the loss of too much lymphocyte material, and the final preparation consisted of 72% viable cells of which 11% were macrophages. Cell suspensions were prepared from the mouse axillary and inguinal lymph nodes, and from the thymus, and were treated similarly. These were purer lymphocyte suspensions, but there was too little material for biochemical analysis.

Mouse Gardner lymphoma cells [20] were grown in the peritoneal cavity of CBA mice, and harvested after 10—14 days. Any erythrocytes present were removed by lysis with 10 mM Tris-HCl as described for the mouse spleen. The cell suspension was prepared similarly. Cell viability was 70%, and the cells were greater than 95% tumour cells.

Electron microscopy

Cells were treated with Karnovsky's fixative [21] at 0°C. They were fixed with 1% OsO_4 in Millonig's buffer [22], washed in water, dehydrated in acetone and propylene oxide and embedded in Spurr resin [23]. Sections were stained with 5% uranyl acetate in 1% acetic acid for ten minutes and Reynold's lead citrate for two to five minutes [24]. They were coated with carbon and examined in a Phillips EM 300 at 60 or 80 kV.

Preparation of cell membranes

The cells were disrupted at 0°C at a cell concentration of about $2 \cdot 10^7$ cells $\cdot$ ml^{-1} by pumping the suspension at 400 ml $\cdot$ h^{-1} through a cell disruption pump [25]. This ruptures the cells but not the subcellular particles by forcing the suspension through a small orifice against a spring-loaded needle at a pressure of about 28 kg $\cdot$ cm^{-2}. The membrane suspension was kept at 0—6°C and was

then subjected to the differential centrifugation scheme used for pig lymphocytes by Allan and Crumpton [26]. Nuclei and unbroken cells were removed by centrifuging at 300 × g_{av} for 15 min, and mitochondria by centrifuging the supernatant at 4000 × g_{av} for 15 min. The post-mitochondrial supernatant was centrifuged at 20 000 × g_{av} for 30 min, giving the plasma membrane sample which was washed once in 10 mM Tris-HCl, pH 7.5 (20°C). The supernatant was retained. The pellets were homogeneously suspended in known volumes of Tris-buffered saline.

Initially the 20 000 × g_{av} pellets were suspended in a few drops of saturated sucrose solution. 5 ml of a continuous gradient 50—20% (w/w) sucrose in 10 mM Tris, pH 7.6 (20°C), was layered on top and the samples centrifuged at 150 000 × g_{av} for 12 h then the band(s) were collected, diluted with 10 mM Tris, centrifuged at 90 000 × g_{av} for 1 h and suspended in 10 mM Tris. Membranes were stored at —70°C. The density of the sucrose immediately above and below the band(s) was calculated from the refractive indices measured in an Abbe' refractometer. The 20 000 × g_{av} surface membrane preparation from the tonsils appeared as a single sharp band and was not appreciably purified by sucrose density gradient centrifugation. 20 000 × g_{av} plasma membrane preparations from leukaemic lymphocytes from 20 ml of blood and Gardner lymphoma cells were treated like those from the tonsil lymphocytes. However, very large quantities of leukaemic lymphocytes were obtained from the cell separator. These were disrupted at about $2 \cdot 10^8$ cells · ml^{-1} and further purified on a continuous sucrose gradient. The purification factors of the marker enzymes after both types of preparation were similar.

Measurement of electron micrographs

The lengths of cell membranes on at least five electron micrographs of each kind of lymphocyte were measured, using an electronic tablet linked to a PDP 11/40 computer. The lengths of the perimeters of the mitochondria were doubled, as they have two membranes, but no allowance was made for the cristae of the mitochondria.

The percentage composition by length was calculated for each type of membrane in the three kinds of cells. The method of calculating the membrane purification factor is given in Discussion.

Enzyme assays

The samples were assayed for the plasma membrane marker 5′-nucleotidase as described by Avruch and Wallach [27], using [^{3}H]adenosine monophosphate, except that the buffer used was 0.1 M glycine/sodium hydroxide, pH 8.5 (20°C), and the enzyme and unused substrate were precipitated with 0.3 ml 5% zinc sulphate and 1 ml 0.15 M barium hydroxide solution.

Succinate dehydrogenase, the mitochondrial marker, was assayed by the method of Pennington [28] as modified by Porteus and Clarke [29]. Glucose-6-phosphatase, the endoplasmic reticulum marker, was assayed by a radioactive method similar to 5′-nucleotidase. In a total volume of 1 ml there was 0.031 M sodium hydrogen maleate/sodium hydroxide buffer, pH 6.5 (20°C), 0.02 mM glucose 6-phosphate with 0.125 μCi [^{14}C]glucose 6-phosphate and up to 0.5 ml sample. Incubation was 30 min at 37°C and the reaction was stopped and the unused substrate precipitated as described for 5′-nucleotidase.

Protein assay

Protein was assayed as described by Lowry et al. [30] using bovine serum albumin as a standard, except that the samples and standards were initially suspended in a total volume of 0.5 ml, containing 5% sodium dodecyl sulphate.

Enzyme purification factors

These were calculated as (activity of enzyme/mg protein in sample)/(activity of enzyme/mg protein in original cell homogenate). If the membrane was subjected to sucrose gradient purification, the factor was calculated in two parts, the purification up to the 20 000 $\times g_{av}$ pellet, and the additional purification obtained from the gradient fraction as compared to the starting material for the gradient, which was kept in 35% sucrose at 4° C while the gradient was centrifuged. This avoided errors due to the preservation of 5′-nucleotidase by sucrose, or the loss of enzyme activity of the other two markers due to decay.

Lipid extraction

The membrane preparations were made up to 0.6 ml in the Tris-buffered saline, and extracted with 0.8 ml methanol and 1.6 ml chloroform, in the proportions recommended by Folch et al. [31]. This method of extracting the wet membrane preparation is believed to be crucial to the phospholipid analysis [18].

Phospholipid analysis

Total phospholipid was assayed as described by McClare [32]. Individual phospholipids were assayed by thin-layer chromatography. The plates were coated with Silica gel 60 F-254, and were bought already prepared from Merck. The spots were visualized with the Dittmer lipid phosphate reagent [33], and measured against known phospholipid standards on a 'flying spot' Vitatron densitometer. Plates were sprayed with water to remove the blue background. A one-dimensional solvent system was first used, which resolved cardiolipin, phosphatidylethanolamine and phosphatidylcholine. The solvent was 7 M aqueous ammonia/methanol/chloroform (1 : 6 : 15.3, by vol.). A two-dimensional system was required to separate sphingomyelin, phosphatidylserine and phosphatidylinositol. The solvent for the first dimension was that described previously, the second was chloroform/methanol/acetic acid/water/acetone (5 : 1 : 1 : 0.5 : 2, by vol.).

Cholesterol was assayed using the enzyme cholesterol oxidase as described by Johnson [18].

Free fatty acids, triglycerides, alkyl diacylglycerides (and cholesterol) were assayed by thin-layer chromatography as described for the phospholipids, except that the solvent system was petroleum ether (b.p. 60—80° C)/diethyl ether/formic acid (25 : 25 : 1, by vol.) and the spots were developed by spraying with a saturated solution of potassium dichromate in 70% sulphuric acid and heating to 140° C for 10 min [34]. The standards were oleic acid, triolein and cholesterol. Results were expressed as mol of oleic acid, triolein or its alkyl diacylglycerides and cholesterol.

Total fatty acids in the lipid extract were methylated using the BF_3 + CH_3O procedure [35] and the H_2SO_4 + CH_3OH procedure [36]. They were subsequently assayed by gas chromatography using a Varian 1400 V Gas Chromatograph with a glass capillary column H 269.

Polyacrylamide gel electrophoresis

A 4—26% 'gradipore' polyacrylamide gradient gel slab was obtained from Universal Scientific Ltd. It was electrophoresed at 50 V for approximately 18 h at room temperature completely immersed in 5 l of an 0.025 M Tris-HCl/0.192 M glycine buffer, pH 8.8 (20°C), containing 0.25% sodium dodecyl sulphate in an Ortec electrophoresis tank. 30 μg membrane protein was dissolved in 3.3% sodium dodecyl sulphate, 20% sucrose and 0.025% bromophenol blue. The mixture was left in boiling water for 1 min before being applied to the gel. The protein was then electrophoresed through the gel at 50 V for another 18 h. The slab gel was fixed for 12 h in a gel stain, containing 2.5 g Coomassie brilliant blue, 908 ml 50% methanol and 92 ml glacial acetic acid/l, and destained electrophoretically in 1 l solution containing 75 ml glacial acetic acid and 50 ml methanol. The electrophoresis method was adapted from Maizel [37]. The gel was calibrated with 2.5 μg of protein standards.

Preparation of liposomes

Mixtures of phospholipid, cholesterol, triolein and diphenyl hexatriene in chloroform/methanol (2 : 1) were evaporated to dryness in a round-bottomed flask under reduced pressure. 8 nmol diphenyl hexatriene were added to 2 mg mixed lipids. The liposomes were prepared in 1 ml Dulbecco's phosphate-buffered saline, without calcium or magnesium [38]. The solution was added to the lipid under nitrogen, and the flasks shaken mechanically for 15 min, as described by Papahadjopoulos and Watkins [39], except that the glass spheres were omitted. Fluorescent labelling of cells was carried out as described by Johnson and Kramers [17]. In general, membranes were prepared from pre-labelled cells, and the diphenyl hexatriene passed through the Folch extract to label the liposomes.

Fluorescence measurements

Fluorescence polarization. Polarization values were read at 37.0 ± 0.3°C using an Elscint MV-1 microviscosimeter. This instrument measures the polarization ratio directly, where

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \tag{1}$$

$I_{\parallel}$ is the intensity of the polarized light emitted parallel to the incident beam, and $I_{\perp}$ the light perpendicular to the beam.

To correct for depolarization due to light scattering, polarization values were plotted against the absorbance of the suspension at 450 nm, and the extrapolated value of the polarization for zero absorbance was taken [16]. The anisotropy, r, was calculated from the relation

$$r = \frac{2P}{3 - P} \tag{2}$$

Fluorescence lifetime measurements. The fluorescent lifetime of the probe was measured directly at 37 ± 0.3°C using an Applied Photophysics nanosecond spectrometer, and Elscint electronics. The light source was a hydrogen or nitrogen arc, run at a pressure of 0.5 atm. The excitation wavelength was

364 nm, and the emitted light was filtered through 3 M $NaNO_2$. No light-scattering corrections were required, and no polarizing filters at an angle of 56° were needed. The best single exponential decay curve was calculated using a computer to deconvolute the result.

Correlation times $\langle\rho\rangle$ were calculated from the Perrin equation, which can be written

$$\frac{r_0}{r} = 1 + \frac{\tau}{\langle\rho\rangle} \tag{3}$$

r_0 is the limiting fluorescence anisotropy when the probe is immobilized, and was taken as 0.362 [40]. τ is the fluorescent lifetime of the probe.

The validity of these measurements as applied to membranes is discussed by Hildenbrand and Nicolau [41].

Results

The percentage of the plasma membrane, mitochondria and endoplasmic reticulum in the original lymphocytes are shown in Table II. The nuclear membranes are not included, as the nuclei were removed from the material in the preliminary centrifugation step. Enzyme purification factors are also shown. The amount of enzyme recovered varied between 80 and 110% for the 5′-nucleotidase and succinate dehydrogenase, but the recovery of glucose-6-phosphatase was only about 60% as it was too dilute to be measured in the supernatant after the 20 000 × g_{av} centrifugation.

TABLE II

PROPORTIONS OF MEMBRANES

Marker enzymes: plasma membrane, 5′-nucleotidase; endoplasmic reticulum, glucose-6-phosphatase; mitochondria, succinate dehydrogenase. The percent membrane is a percent by length, see text. CLL, chronic lymphocytic leukaemia.

	% membrane in cell	4000 × g_{av}		Plasma membrane preparation	
		Marker enzyme purification factor	% membrane in pellet	Marker enzyme purification factor	% membrane in pellet
Human tonsil lymphocyte					
Plasma membrane	55 ± 13	2.2 ± 0.5 (5)	51	5.9 ± 0.8 (4)	85
Endoplasmic reticulum	22 ± 9	0.2 ± 0.2 (5)	3	1.2 ± 0.7 (5)	7
Mitochondria	23 ± 16	4.7 ± 0.4 (5)	46	1.4 ± 0.6 (5)	8
Human CLL lymphocyte	54 ± 16	2.6 ± 1.3 (4)	45	5.5 ± 0.2 (4)	74
Endoplasmic reticulum	9 ± 4	0.9 ± 0.3 (2)	3	2.2 ± 0.5 (2)	5
Mitochondria	36 ± 16	4.5 ± 1.0 (4)	52	2.3 ± 1.0 (4)	21
Mouse Gardner lymphoma					
Plasma membrane	33 ± 10	1.8 ± 0.5 (4)	33	7.9 ± 0.8 (3)	75
Endoplasmic reticulum	28 ± 13	0.5 ± 0.2 (2)	8	1.2 ± 0.2 (2)	10
Mitochondria	39 ± 7	2.8 ± 0.7 (4)	60	1.4 ± 0.6 (3)	16

Calculation of the percentage of plasma membrane in each preparation

The specific activity of each marker enzyme is increased by the removal of the nuclei and cytosol. For the Gardner lymphoma, this was 2.6 for the 5'-nucleotidase and succinate dehydrogenase. The specific activity of each membrane marker enzyme would then increase, or decrease, as the membranes were partially separated by differential centrifugation.

The percentage of plasma membrane in a preparation was calculated from

$$\frac{f_p l_p \times 100}{f_p l_p + f_m l_m + f_e l_e}$$

where f_p, f_m, and f_e were the purification factors for the plasma, mitochondrial and endoplasmic reticulum marker enzymes, respectively, and l_p, l_m and l_e the lengths of the membranes in the original cell. 10% of the nucleotidase was recovered in the $300 \times g_{av}$ pellet, 30—40% in the $4000 \times g_{av}$ pellet, 30—20% in the $20\,000 \times g_{av}$ pellet and 30% in the supernatant. Further data on the tonsil lymphocyte 5'-nucleotidase and cholesterol is given in Johnson and Nicolau [16].

Table III gives the proportions of the phospholipids in the extracted plasma membrane preparations and the phospholipids in the model liposome preparation. The phospholipid composition of the three membranes is very similar. No lysolipids were detected. Proportions of the lipids other than phospholipids in the Folch extracts are shown in Table IV, and here there are significant differences between the samples. The three chronic lymphocytic leukaemia samples varied among themselves, from extremely low triglycerides and other glycerides to values similar to those obtained for tonsil lymphocytes. One sample had a greatly raised fatty acid content. The Gardner lymphoma showed a progressive increase in glycerides and cholesterol as the tumour was successively passaged, hence the large standard deviation in Tables IV and V.

The esterified and free fatty acids in the Folch extract were also analysed, and the relevant data are stored in the BBA data bank. Those of the tonsil and chronic lymphocytic lymphocytes are very similar to each other, and those of the Gardner lymphoma similar to the model phospholipid used in the fluores-

TABLE III

PERCENT PHOSPHOLIPIDS IN PLASMA MEMBRANE PREPARATIONS

The phospholipids of the model membrane extract were taken from a previous unpublished analysis of pig lymphocyte plasma membranes. CLL, chronic lymphocytic leukaemia.

	Tonsil	CLL	Mouse lymphoma	Model membrane
Cardiolipin	<0.5	<0.5	1.1	—
Phosphatidylcholine	48.4	50.4	45.6	45.8
Phosphatidylethanolamine	23.7	23.0	24.7	28.7
Phosphatidylinositol	11.1	7.4	13.6	—
Phosphatidylserine	10.6	10.4	11.4	12.5
Sphingomyelin	6.2	8.8	3.5	12.9
% recovery	109	92	97	

TABLE IV

TOTAL GLYCERIDES, TRIGLYCERIDES AND FREE FATTY ACIDS IN PLASMA MEMBRANE PREPARATIONS AS A MOLAR RATIO OF PHOSPHOLIPID

The cholesterol/phospholipid ratio for the Gardner lymphoma plasma membrane increased from 0.30 to 0.45 as the glycerides increased. CLL, chronic lymphocytic leukaemia.

Lymphocyte	Total glycerides	Triglycerides	Free fatty acids
Human			
Tonsil (3)	0.079	0.030	0.026
Range	0.040—0.119	0.022—0.043	0.010—0.055
CLL (3)	0.051	0.009	0.131
Range	0.021—0.080	0.002—0.028	0.055—0.28
Mouse			
Spleen	0.030	0.011	0.042
Gardner lymphoma (4)	0.179	0.053	0.109
Range	0.015—0.295	0.004—0.11	0.096—0.13

cence experiments. The percentage of saturated acids is similar in all four samples, and differences in fluidity cannot be attributed to changes in the composition of the phospholipid fatty acids.

TABLE V

P values for the human tonsil and chronic lymphocytic leukaemia (CLL) cells, membranes and liposomes were taken from Johnson and Kramers [17]. Polarization values are the mean of at least five samples, S.D. ± 5%, fluorescence lifetimes were measured on three samples, S.D. ± 0.2 ns. To facilitate comparison of these results with those measured at other temperatures, we found the polarization of the tonsil plasma membrane increased linearly by 0.0033/°C between 40 and 3°C. The biochemical analytical results for the cholesterol/phospholipid molar ratio are shown ± S.D. with the number of determinations in parenthesis. The cholesterol/phospholipid ratio for the pure plasma membrane was calculated from the biochemical analysis data and the percent of plasma membrane in the plasma membrane preparation (Table II). DPH, diphenyl hexatriene. PM, plasma membrane.

Lymphocyte	Sample	*P*	τ	$\langle\rho\rangle$	Plasma membrane cholesterol/phospholipid molar ratio		
					Biochemical	DPH fluorescence	Pure membrane (calculated)
Human tonsil	Whole cell	0.214	7.95	5.9	—	—	
	PM preparation	0.244	8.6	8.2	—	—	
	PM liposome	0.214	7.75	5.7	0.42 ± 0.03 (6)	0.42	0.49
Human CLL	Whole cell	0.224	7.8	6.2	—	—	
	PM preparation	0.248	8.3	8.2	—	—	
	PM liposome	0.205	8.2	5.6	0.37 ± 0.04 (5)	0.375	0.50
Mouse spleen	Whole cell	0.214	7.9	5.85	—	—	
	PM preparation	0.245	8.0	7.7	—	—	
	PM liposome	0.226	8.3	6.8	0.41 ± 0.02 (2)	0.425	—
Mouse Gardner lymphoma	Whole cell	0.168	6.9	3.3	—	—	
	PM preparation	0.193	8.0	4.9	—	—	
	PM liposome	0.175	7.3	3.8	0.34 ± 0.07 (7)	0.38	0.45
Mouse thymus	Whole cell	0.203	7.7	5.1			
Mouse lymph node	Whole cell	0.201	7.65	5.0			

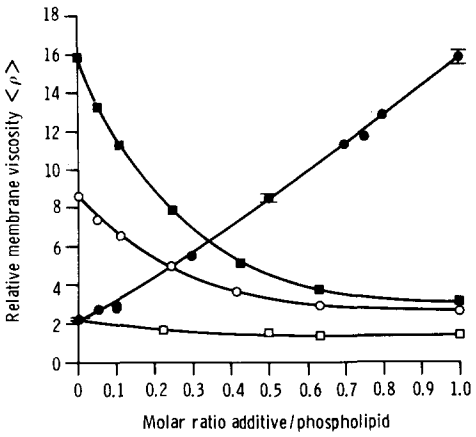

Fig. 1. The correlation coefficient or relative membrane viscosity for model phospholipid, cholesterol and triglyceride membranes. The phospholipid composition is given in Table III. ●, phospholipid + increasing mol ratio of cholesterol. The bars represent the S.D. on five different preparations; □, phospholipid + increasing mol ratio of triolein; ○, phospholipid : cholesterol (2 : 1) + increasing mol ratio of triolein; ■, phospholipid : cholesterol (1 : 1) + increasing mol ratio of triolein. Note the effect of triolein in counteracting the stiffening effect of cholesterol.

Fluorescence polarization measurements

The correlation times or relative membrane viscosities of the model phospholipid liposomes with or without cholesterol or triolein are shown in Fig. 1. It will be seen that quite small amounts of triolein in cholesterol-containing liposomes apparently reverses the effect of the sterol. Table V shows the fluorescence polarization, lifetime and correlation time for diphenyl hexatriene in whole lymphocytes, the plasma membrane preparations and the Folch extracts of the plasma membranes. If an allowance is made for the amount of glycerides present in the Folch extract (Table III) and the 2 : 1 phospholipid : cholesterol against triolein curve in Fig. 1, the cholesterol : phospholipid ratio can be determined from the standard curve obtained from the model lipids. The figures are in excellent agreement with those obtained analytically.

If it is assumed that all cholesterol is in the plasma membrane, the cholesterol/phospholipid ratio in the pure membrane can be calculated by dividing the cholesterol/phospholipid ratio for a given preparation by the proportion of plasma membrane present in that preparation (see Table II).

Correlation times for diphenyl hexatriene in normal mouse thymocytes and lymph node lymphocytes were also measured, and are shown in Table V. They are very similar to that obtained for the splenic lymphocytes + macrophages.

Fig. 2 shows the lymphocyte membrane proteins from both the $4000 \times g_{av}$ and the plasma membrane preparations. If one is interested in a particular protein, it is possible to tell if it is greatly enriched in the plasma membrane, and whether it is found in all four types of lymphocyte. The densities of the plasma membranes were 1.180, 1.152, and 1.149 $g \cdot ml^{-1}$ for the tonsil, leukaemia and Gardner lymphoma, respectively.

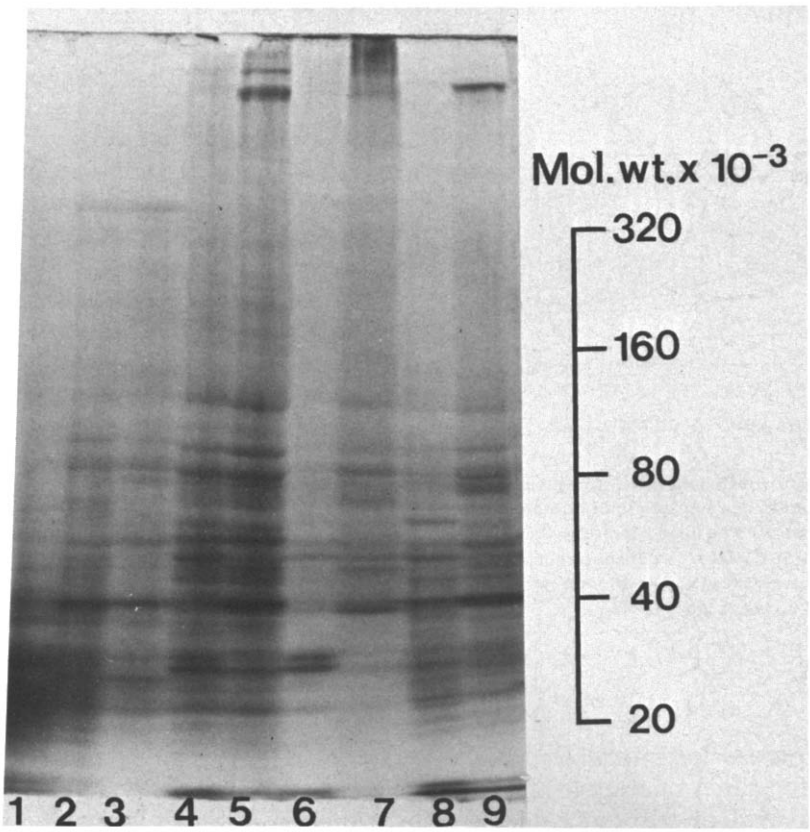

Fig. 2. Gradient gel electrophoresis of membrane proteins: 1, 4000 × g_{av} preparation of human chronic lymphocytic lymphocytes; 2, and 3, plasma membrane preparation of human chronic lymphocytic lymphocytes; 4 and 5, 4000 × g_{av} and plasma membrane preparation of mouse Gardner lymphoma lymphocytes; 6 and 7, 4000 × g_{av} and plasma membrane preparation of mouse splenic lymphocytes; 8 and 9, 4000 × g_{av} and plasma membrane preparation of human tonsil lymphocytes.

Discussion

Membrane proteins

From Table V, it will be seen that there is a difference of 2.5—2.6 in the relative membrane viscosity $\langle \rho \rangle$ of the human lymphocyte plasma membranes and their lipid liposomes, whereas the difference between mouse lymphocyte plasma membranes and their liposomes is 0.9—1.1. This shows that the motion of the probe is more restricted by the human lymphocyte plasma membrane proteins than by those of the mouse. The plasma membrane density figures suggest that there is rather more protein in general associated with the human lymphocyte plasma membranes but there must be appreciably more in the hydrophobic region of the plasma membrane.

Van Blitterswijk et al. [43] found no difference between the polarisation of their enzymically uncharacterized mouse thymocyte and T-leukaemia plasma membrane preparations and the liposomes prepared from them, but they dried

their membranes before extraction, a process which Johnson [18] has shown to result in loss of phospholipid producing a spuriously high cholesterol/phospholipid ratio which would mask the loss of the stiffening effect of the protein.

The electrophoresis gel shows a major protein band in the plasma membrane preparations, with a molecular weight of about 40 000. Barber and Crumpton [44] have identified this as actin, molecular weight 41 700. As reported by Owen et al. [45] it is particularly prominent in preparations from B lymphocytes. Demus [1], studying tonsil lymphocytes, did not find a strong band in this region, and the density of his plasma membrane preparations were less than $1.14\ g \cdot ml^{-1}$ (33% sucrose), as opposed to our figure of $1.18\ g \cdot ml^{-1}$. Marique and Hildebrand [4] also obtained a low density for their chronic lymphocytic leukaemia plasma membranes, $1.115\ g \cdot ml^{-1}$. Our figure was $1.15\ g \cdot ml^{-1}$. It seems possible that actin may be removed from the plasma membrane if an homogenizer is used to disrupt the lymphocytes.

Membrane lipids

Specific cholesterol assays and fluorescence depolarization measurements with diphenyl hexatriene showed that all the mouse and human lymphocyte plasma membranes had lower cholesterol/phospholipid ratios than had generally been reported previously.

The cholesterol/phospholipid ratio for the chronic lymphocytic leukaemic cell plasma membranes reported in this paper agrees well with that reported by Marique and Hildebrand [4], the only authors on Table I to extract their membrane preparation without drying it first. Johnson [18] found that phospholipid was lost as the membrane preparation dried, giving a spuriously high cholesterol/phospholipid ratio. The other authors, with the exception of Pratt et al. [5] whose results are outside both limits, found cholesterol/phospholipid ratios of 0.69—1.02 for normal or leukaemic cells from mouse, rat or man. Cholesterol/phospholipid ratios as high as this would require diphenyl hexatriene plasma membrane correlation times of 13—18. Pessin et al. [46] and van Hoeven et al. [47] have both reported increased fluidity in their cells due to triglycerides in vesicles, but the polarisation and analytical results for the Gardner lymphoma show that in this case the glyceride occurs in the plasma membrane.

From Table II it can be calculated that the purification factor for 5′-nucleotidase in pure chronic lymphocytic lymphocytes or tonsil lymphocyte plasma membranes should be about 7, and about 10 for the Gardner lymphoma. Most of the authors in Table I measure much higher values than this. However, 5′-nucleotidase is not evenly distributed among the lymphocytes themselves. Quagliata et al. [48] have shown that it is low in most cases of chronic lymphocytic leukaemia lymphocytes, and Rowe et al. [49] have shown that human B lymphocytes have 3.5 times as much 5′-nucleotidase than on T cells, although even then only about 33% of the B cells stain positively for 5′-nucleotidase, a result we have confirmed for tonsil cells (Johnson, S.M. and Edwards, A.J., unpublished data). Thus the high purification factors obtained by many authors in Table I may represent the product of a cell selection process and a plasma membrane purification, and this is particularly likely to happen if the percentage yield is low.

It will be noted that the results in this paper do not confirm the conclusions of Inbar and coworkers [11—14]. There was no difference in the relative membrane fluidity for human tonsil and chronic lymphocytic lymphocytes, nor did their cholesterol/phospholipid ratios differ. The mouse Gardner lymphoma cell and plasma membrane were more fluid than the normal mouse splenic lymphocyte and plasma membrane, but this was due to a raised proportion of triglycerides and alkyl diacylglycerides in the lymphoma rather than to a lowered proportion of cholesterol.

The normal and pathological lymphocytes studied here were all very similar in their cholesterol/phospholipid ratios, and in their phospholipid composition. The fatty acids of the two human lymphocyte cell membranes were very similar. The gradient electrophoresis gel showed that actin was the most prominent protein in 3/4 of the plasma membranes, and enabled interlymphocyte comparisons to be made. The most interesting differences are shown in Table IV where the proportions of free fatty acids and glycerides varied widely, and may give useful clues to metabolic differences between the normal and abnormal lymphocytes.

Acknowledgements

We would like to thank H. Behlau, of the Institut für Strahlenchemie, Max-Planck-Institut für Kohlenforschung for the fatty acid analysis of the phospholipids. We would like to thank Mrs. Ellis and Dr. S. Patterson for their help with the electron microscopy.

References

1 Demus, H. (1973) Biochim. Biophys. Acta 291, 93—106
2 Allan, D. and Crumpton, M.J. (1972) Biochim. Biophys. Acta, 274, 22—27
3 Crumpton, M.J. and Snary, D. (1974) Preparation and Properties of Lymphocyte Plasma Membrane, in Contemporary Topics in Molecular Immunology, Vol. 3, pp. 27—56, Plenum Press
4 Marique, D. and Hildebrand, J. (1973) Cancer Res. 33, 2761—2767
5 Pratt, H.P.M., Saxon, A. and Graham, M.L. (1978) Leuk. Res. 2, 1—10
6 Ladoulis, C.T., Misra, D.N., Estes, L.W. and Gill, III T.J. (1974) Biochim. Biophys. Acta 356, 27—35
7 Smith, W.I., Ladoulis, C.T., Misra, D.N., Gill III T.J. and Bazin, H.H. (1975) Biochim. Biophys. Acta 382, 506—525
8 Misra, D.N., Ladoulis, C.T., Estes, L.W. and Gill, III T.J. (1975) Biochemistry 14, 3014—3024
9 Dods, R.F., Essner, E. and Barclay, M. (1972) Biochem. Biophys. Res. Commun. 46, 1074—1081
10 Warley, A. and Cook, G.M.W. (1973) Biochim. Biophys. Acta 323, 55—68
11 Inbar, M., Shinitzky, M. and Sachs, L. (1974) FEBS Lett. 38, 268—270
12 Inbar, M. and Shinitzky, M. (1974) Proc. Natl. Acad. Sci. U.S. 71, 4229—4231
13 Shinitzky, M. and Inbar, M. (1974) J. Mol. Biol. 85, 603—615
14 Inbar, M. (1976) FEBS Lett. 67, 180—185
15 Pagano, R.E., Ozato, K. and Ruysschaert, J.M. (1977) Biochim. Biophys. Acta 465, 661—666
16 Johnson, S.M. and Nicolau, C. (1977) Biochem. Biophys. Res. Commun. 76, 869—874
17 Johnson, S.M. and Kramers, M. (1978) Biochem. Biophys. Res. Commun. 80, 451—457
18 Johnson, S.M. (1979) Anal. Biochem. 95, 344—350
19 Boyle, W. (1978) Transplantation 6, 761—764
20 Gardner, W.U.,Dougherty, T.F. and Lane Williams, W. (1974) Cancer Res. 4, 73—87
21 Karnovsky, M.J. (1965) J. Cell Biol. 27, 137—138A
22 Millonig, G. (1961) J. Biophys. Biochem. Cytol. 11, 736—739
23 Spurr, A.R. (1966) J. Ultrastruct. Res. 26, 31—43
24 Reynolds, E.S. (1963) J. Cell Biol. 17, 208—212
25 Wright, B.M., Edwards, A.J. and Jones, V.E. (1974) J. Immunol. Methods 4, 281—296
26 Allan, D.A. and Crumpton, M.J. (1970) Biochem. J. 120, 133—143

27 Avruch, J. and Wallach, D.F.H. (1971) Biochim. Biophys. Acta 233, 334—347
28 Pennington, R.J. (1961) Biochem. J. 80, 649—654
29 Porteus, J.W. and Clarke, B. (1965) Biochem. J. 96, 159—171
30 Lowry, O.H., Rosenbough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
31 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1956) J. Biol. Chem. 226, 497—509
32 McClare, C.W.F. (1971) Anal. Biochem. 39, 527—530
33 Dittmer, J. and Lester, R. (1964) J. Lipid Res. 5, 126—127
34 Blank, M.L., Schmit, J.A. and Privett, O.S. (1964) J. Am. Oil Chem. Soc. 41, 371—376
35 Metcalfe, L.D., Schmitt, A.A. and Pelka, J.A. (1966) Anal. Chem. 38, 514—520
36 Rogozinski, M. (1964) J. Gas Chromatogr. 2, 136—150
37 Maizel, J.V. (1972) in Methods in Virology, (Maramorosch, K. and Koprowski, H., eds.), Vol. 5, Academic Press, New York and London
38 Dulbecco, R. and Vogt, P. (1954) J. Exp. Med. 99, 167—182
39 Papahadjopoulos, D. and Watkins, J.C. (1967) Biochim. Biophys. Acta 135, 639—652
40 Shinitzky, M. and Barenholz, Y. (1974) J. Biol. Chem. 249, 2652—2657
41 Hildenbrand, K. and Nicolau, C. (1979) Biochim. Biophys. Acta 553, 365—377
42 Snyder, F. and Snyder, C. (1975) Prog. Biochem. Pharmacol. 10, 1—41
43 Van Blitterswijk, W.J., Emmelot, P., Hilkmann, H.A.M., Oomen-Meurlemans, E.P.M. and Inbar, M. (1977) Biochim. Biophys. Acta 467, 309—320
44 Barber, B.H. and Crumpton, M.J. (1976) FEBS Lett. 66, 215—220
45 Owen, M.J., Auger, J., Barber, B.H., Edwards, A.J., Walsh, F.S. and Crumpton, M.J. (1978) Proc. Natl. Acad. Sci. U.S. 75, 4484—4488
46 Pessin, J.E., Salter, D.W. and Glaser, M. (1978) Biochemistry 17, 1997—2004
47 Van Hoeven, R.P., van Blitterswijk, W.J. and Emmelot, P. (1979) Biochim. Biophys. Acta 551, 44—54
48 Quagliata, F., Faig, D., Conklyn, M. and Silber, R. (1974) Cancer Res. 34, 3197—3202
49 Rowe, M., de Gast, C.G., Platts-Mills, T.A.E., Asherson, G.L., Webster, A.D.B. and Johnson, S.M. (1979) Clin. Exp. Immunol. 36, 97—101