Lipid patterns in human leukocytes maintained in long-term culture

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ABSTRACT The lipid composition of leukocytes maintained in long-term culture was examined in order to clarify the role of immaturity in previously observed differences between normal mature leukocytes and leukemic cells. Cell cultures derived from three types of leukocytes were examined: normal lymphocytes, Burkitt lymphoma, and chronic myelocytic leukemia. Lipid extracts were analyzed for total lipid weight, phospholipids, neutral lipids, and glycolipids. Distribution of individual phospholipids was determined by quantitative two-dimensional thin-layer chromatography. The main phospholipids were phosphatidylcholine (51–54%) and phosphatidylethanolamine (24–25%), with smaller amounts of phosphatidylinositol, phosphatidylserine, sphingomyelin, and cardiolipin. All three types of cultured cells showed a remarkable similarity in total phospholipid content ($17–18 \times 10^{-15}$ moles/cell) as well as in phospholipid distribution. More variation was seen in neutral lipid content. Glycolipid was abundant (17–23% of total lipid weight) and was present mostly as ceramide dihexoside. Compared with normal lymphocytes or polymorphonuclear leukocytes, the cultured cells showed increased phosphatidylcholine, decreased sphingomyelin, and decreased cholesterol content, similar to the changes found in leukemic leukocytes. These findings suggest that the altered lipid patterns found in leukemic leukocytes are a reflection of cell immaturity rather than a characteristic peculiar to the leukemic state.

SUPPLEMENTARY KEY WORDS tissue culture · Burkitt lymphoma · leukemia · phospholipids · glycosphingolipids · cholesterol · thin-layer chromatography

HUMAN LEUKOCYTES of different morphological types are known to differ also in lipid composition. Differences in lipid patterns have been demonstrated not only between normal human lymphocytes and polymorphonuclear leukocytes, but also between mature normal cells and leukemic leukocytes of the same apparent morphological series (1). In a previous study, it was found that leukemic cells, in comparison with normal lymphocytes or polymorphonuclear leukocytes, contain relatively more phosphatidylcholine, less sphingomyelin, and less cholesterol. If the observed changes in lipid patterns are a manifestation of cell immaturity rather than a specific characteristic of the leukemic state, similar changes may be expected to occur in undifferentiated cultured leukocytes as well.

The commercial availability of quantities of human leukocytes maintained in long-term tissue culture offers new opportunities for studies of structure, immunochemical properties, and metabolism of leukocytes. Such studies can be properly interpreted, however, only when the relationship between cultured cells, leukemic cells, and normal circulating human leukocytes has been more clearly defined.

In the present study, three different strains of human leukocytes maintained in long-term culture, one derived from normal lymphocytes and two from abnormal cell types, were found to have a remarkably similar pattern of phospholipid composition. In certain respects, the characteristic lipid pattern of these undifferentiated cultured cells resembled that of leukemic cells more closely than that of normal mature lymphocytes or polymorphonuclear leukocytes.

METHODS

Preparation of Leukocyte Suspensions

Viable human cell lines maintained in continuous long-term tissue culture for periods of 2–6 yr were obtained from Associated Biomedic Systems, Inc., Buffalo, N.Y., in lots of 10^9 cells. The cells had been grown in a culture medium of RPMI 1640 plus 10% fetal calf serum. Lot RPMI 1788 consisted of cells derived from normal

lymphocytes obtained from peripheral blood, with antigenic specificity HL-A_2, HL-A_6, HL-A_7, and warranted to be free of EB virus. Lot P3J was derived from a case of Burkitt lymphoma and was known to contain EB virus. Lot RPMI 6410 was derived from a case of chronic myelocytic leukemia. On arrival, cell samples were examined immediately for viability and morphology; washing and extraction were completed within 5 hr from the time of shipment by the vendor.

Blood specimens containing normal or leukemic leukocytes were obtained from normal volunteers and patients of the Bronx Municipal Hospital Center and The New York Hospital. Isolation of leukocytes from whole blood, separation of cell types, and evaluation of purity and viability were performed as previously described (1).

Each cell suspension was washed 3–4 times with Hanks' balanced salt solution, pH 7.4, and cells were collected after each washing by centrifugation at 200 *g* for 10 min at 20°C. A small amount of disodium EDTA was added just before each centrifugation (final concentration 0.05%) to inhibit irreversible clumping. Viability of the washed cells averaged 95% by the method of trypan blue exclusion (2). Total cell counts were determined with an electronic cell counter (Coulter, model F). Siliconized or plastic vessels were used for handling intact cells before extraction of the lipids.

Extraction and Analysis of Lipids

Lipids were extracted from the cell samples immediately after the final washing. Redistilled reagent grade methanol and chloroform, deaerated with nitrogen and cooled to 5°C just before use, were employed for extraction by the procedure described previously (1) in a final proportion of chloroform to methanol of 2:1 (v/v). Extracts were filtered through sintered glass funnel and were washed by the method of Folch, Lees, and Sloane Stanley (3), with 0.1 M potassium chloride solution in the upper phase. The lower phase was taken nearly to dryness in a rotary evaporator at 37°C, and the lipid was redissolved in cold chloroform–methanol 2:1 (v/v), filtered again, and stored at −20°C in tightly stoppered amber bottles.

Total lipid weight was determined with a Cahn Electrobalance by the procedure described previously (1). Lipid phosphorus was determined by a modification (1) of the method of Beveridge and Johnson (4). Glycolipids were separated from the lipid extracts by the column chromatographic procedure of Vance and Sweeley (5), and the total glycolipid content was estimated gravimetrically. Neutral lipids were separated by thin-layer chromatography on plates of Silica Gel H (E. Merck A.G., Darmstadt, Germany) with a solvent system of hexane–diethyl ether–glacial acetic acid 90:10:7.5 (v/v/v) (6) (Fig. 1). Cholesterol and cholesteryl esters were determined by the ferric chloride method of Leffler (7). Triglycerides were estimated by ester analysis (8). Plasmalogens were estimated as α,β-unsaturated ethers by the spectrophotometric iodination method of Gottfried and Rapport (9).

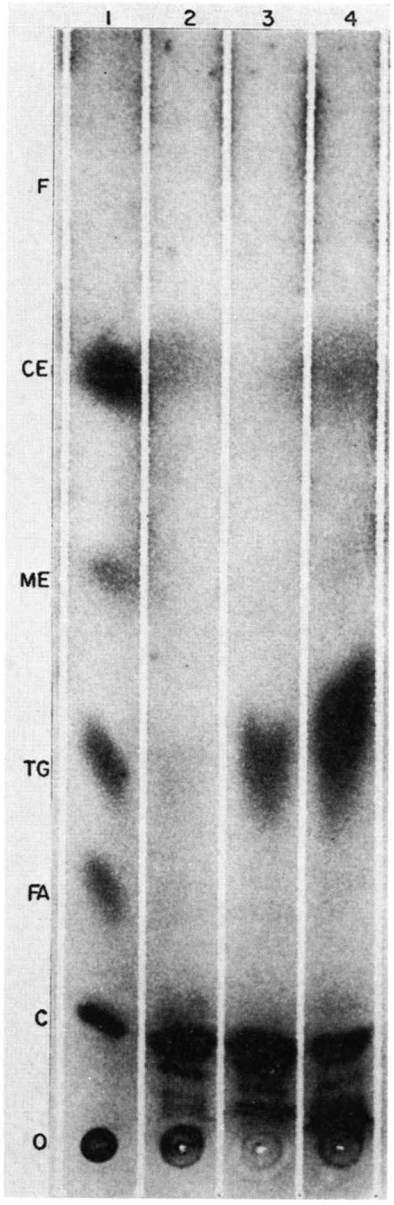

FIG. 1. Thin-layer chromatogram of neutral lipids of leukocytes maintained in tissue culture. Procedure is described in text. Lane *1*, reference lipid mixture: cholesteryl oleate, methyl oleate, triolein, oleic acid, and cholesterol, 5 μg each; lane *2*, cultured normal lymphocytes, 450 μg of total mixed lipid extract; lane *3*, myelocytic leukemia cells, 450 μg of lipid; lane *4*, Burkitt lymphoma cells, 450 μg of lipid. Abbreviations: *F*, solvent front; *CE*, cholesteryl esters; *ME*, fatty acid methyl esters; *TG*, triglycerides; *FA*, free fatty acids; *C*, free cholesterol; *O*, origin.

Phospholipids were separated by thin-layer chromatography on Silica Gel H (Merck). Major phospholipid groups were separated by one-dimensional chroma-

tography (10) with a solvent system of chloroform–methanol–glacial acetic acid–water 25:15:2:2 (v/v/v/v), and estimates of relative phospholipid distribution were made by analysis in duplicate of the phosphorus content of individual spots (1). Recovery of phosphorus from these chromatograms averaged 96% of the total lipid phosphorus in the starting samples. For a more detailed analysis, two-dimensional thin-layer chromatography was employed (Fig. 2). Thin-layer chromatoplates of 0.25-mm nominal thickness were prepared with Silica Gel H suspended in 0.001 M sodium carbonate (10). Solvent I consisted of chloroform–methanol–glacial acetic acid–water 25:15:2:2, with 0.01% α-tocopherol added to inhibit oxidative degradation of lipids. Solvent II was composed of diisobutyl ketone–glacial acetic acid–water 40:25:5 (11). Lipid spots were detected either by exposure to iodine vapor or by charring at 180°C after the plates had been sprayed with a 20% aqueous solution of ammonium bisulfate (12). The identity of individual lipid spots was determined as described previously (1). In addition, glycolipid spots were identified with an α-naphthol spray reagent (13). Standard reference lipids were obtained from Applied Science Laboratories, State College, Pa., and from Supelco, Inc., Bellefonte, Pa.

RESULTS

The cell lines used in this study, obtained from a commercial supplier, had been maintained in long-term culture by a method similar to that described by Moore e al. (14 . The cell lines examined were derived from (*a*) normal human lymphocytes from peripheral blood, (*b*) Burkitt lymphoma, and (*c*) chronic myelocytic leukemia. All three cell populations consisted mostly of cells with a characteristic "blastoid" morphology resembling lymphoblasts.

Each of the three types of cultured cells contained a substantial amount of lipid, ranging from 18 to 27 pg/cell (Table 1). Phospholipid constituted the major lipid class in each case, amounting to 52–67% of the total lipid weight. Although the relative proportions varied, the absolute content of phospholipid was remarkably uniform in all three cell lines, ranging only from 17.2 to 17.9 × 10^{-15} moles/cell (Table 2). This quantity of phospholipid is much larger than that encountered in normal mature leukocytes or even in most leukemic leukocytes, though comparable levels were previously found in the large monoblastic cells of a case of acute monocytic leukemia (1).

The relative phospholipid distribution in each of the cell types is shown in Table 2. Here, too, there is a high degree of similarity between the three kinds of cultured cells, compared with other leukocytes. Phosphatidyl-

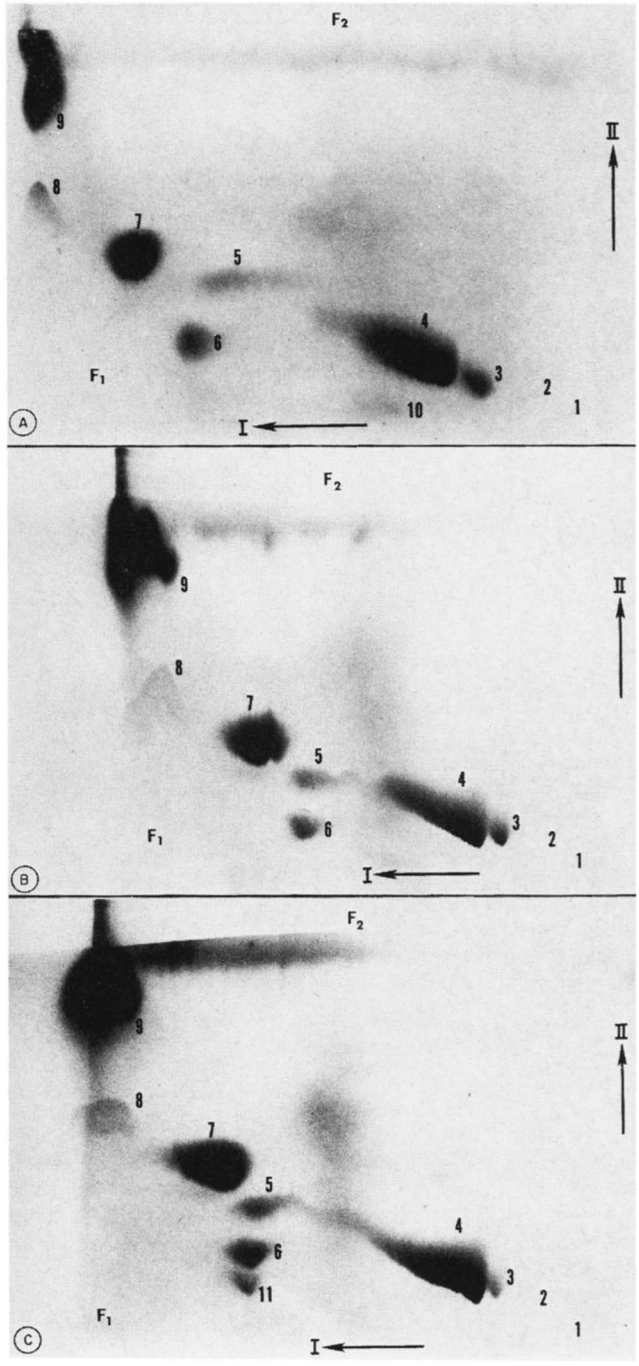

FIG. 2. Two-dimensional chromatograms of lipids of leukocytes maintained in tissue culture. Procedure and solvent systems are described in text. For each chromatogram, 450 μg of total mixed lipid extract was used. Lipid spots were visualized by exposure to iodine vapor. *A*, cultured normal lymphocytes; *B*, myelocytic leukemia cells; *C*, Burkitt lymphoma cells. Key: *1*, origin; *2*, lysolecithin; *3*, sphingomyelin; *4*, phosphatidylcholine; *5*, phosphatidylserine; *6*, phosphatidylinositol; *7*, phosphatidylethanolamine; *8*, cardiolipin; *9*, neutral lipids and phosphatidic acid; *10*, unidentified glycolipid; *11*, ceramide dihexoside. (Other glycolipids are present but not visible in these figures.) Numerals *I* and *II* designate direction of flow with solvent systems I and II, with solvent fronts F_1 and F_2, respectively.

TABLE 1 MAJOR LIPID CLASSES OF HUMAN LEUKOCYTES MAINTAINED IN LONG-TERM CULTURE

Cell Type	Total Lipid Wt	Cholesterol		Phospho-lipid†	Trigly-ceride‡	Glycolipid§
		Free	Esterified*			
	pg/cell	*% of total lipid weight in major lipid classes*				
Cultured normal lymphocytes (RPMI 1788)	19.6	11.3	2.9	64.5	3.4	17.9
Burkitt lymphoma (P3J)	27.0	7.2	2.0	52.3	15.1	23.4
Myelocytic leukemia (RPMI 6410)	18.2	8.5	1.5	67.2	5.5	17.3

* Calculated as cholesteryl oleate.
† Estimated from total lipid P, assuming average mol wt = 775.
‡ Calculated as triolein.
§ Determined by direct weight of isolated glycolipid fraction (5), corrected for residual phospholipid.

TABLE 2 PHOSPHOLIPID DISTRIBUTION OF HUMAN LYMPHOID AND MYELOID CELLS

Cell Type	Phospholipid	Phospholipid Distribution								C/P Ratio	
		PC	PE	SPH	PS	PI	CL	PA	LL	C/Total P	C/PC
	moles × 10^{-16}/cell	*mole %**								*molar ratio*	
Lymphoid cells											
Cultured normal lymphocytes (RPMI 1788)	17.2	51.3	24.0	4.8	6.0	7.8	4.7	0.4	1.0	0.41	0.79
Cultured Burkitt lymphoma cells (P3J)	17.9	54.0	24.8	3.9	3.8	7.1	4.9	1.2	0.3	0.30	0.56
Acute lymphocytic leukemia (n = 3)	4.28	48.7	26.2	6.6	3.2	9.4		5.3	0.5	0.25	0.52
Chronic lymphocytic leukemia (n = 6)†	4.00	49.5	26.6	6.6	5.9	7.3		3.9	0.3	0.38	0.78
Normal lymphocytes (n = 8)†‡	4.73	43.6	28.7	10.1	13.4			3.2	1.0	0.60	1.39
	±0.33	±0.7	±0.8	±0.9	±0.6			±0.8	±0.4	±0.03	±0.08
Myeloid cells											
Cultured myelocytic leukemia cells (RPMI 6410)	17.3	52.8	25.1	4.4	5.5	7.7	3.2	0.2	1.0	0.27	0.51
Acute myelocytic leukemia (n = 5)	13.6	47.4	28.2	9.0	4.9	6.7	2.9	0.3	0.6	0.32	0.68
Chronic myelocytic leukemia (n = 3)	11.1	40.7	30.9	10.3	6.6	7.4	2.0	0.4	1.5	0.46	1.03
Normal polymorphonuclear leukocytes (n = 5)†‡	9.67	38.6	33.4	10.5	15.0			1.3	1.1	0.54	1.41
	±0.50	±0.8	±0.5	±0.3	±0.5			±0.1	±0.4	±0.01	±0.04

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; SPH, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol; CL, cardiolipin; PA, phosphatidic acid; LL, lysolecithin; C/P ratio, cholesterol/lipid phosphorus, molar ratio.
* Mean values.
† Ref. 1.
‡ ± Standard error.

choline was the main component and made up 51–54% of the total phospholipid in the cultured cells, compared with only 39% for normal polymorphonuclear leukocytes and 44% for normal mature lymphocytes ($P < 0.01$). At the same time, the sphingomyelin content was only 4–5% in the cultured cells, compared with 10% in normal mature cells.[1] Similar alterations of phosphatidylcholine and sphingomyelin content were present in leukemic cells, though these changes were of smaller magnitude. An additional constant finding in the cultured cells was a moderate reduction in phosphatidylethanolamine content to 24–25%, compared with 29–33% in mature cells.[2] No notable alterations were observed in the other phospholipids. The plasmalogen content, as in normal mature lymphocytes (1), was fairly small, with an α,β-unsaturated ether:lipid phosphorus molar ratio of 0.12–0.13 for all three cell types.

In contrast to the similarity of phospholipid patterns in the cultured cells, there was much less uniformity among the other lipid classes (Table 1 and Fig. 1). Free fatty acid was present only in trace amounts. Triglyceride was found in small amounts in cultured lymphocytes and myelocytic leukemia cells, but it constituted 15% of the total lipid of Burkitt lymphoma cells. Cholesterol, which constituted 9–14% of the total cell lipid, was present mostly in nonesterified form.

Proportionately smaller amounts of cholesterol were found in the cultured cells than in mature lymphocytes or polymorphonuclear leukocytes; the cholesterol–phospholipid (C/P) molar ratios for the cultured cells were only 0.27–0.41, compared with values of 0.60 and 0.54 in normal mature lymphocytes and polymorphonuclear leukocytes, respectively. A similar decrease in

[1] For cultured lymphocytes, $P < 0.10$; for lymphoma, $P < 0.05$; and for myelocytic leukemia cells, $P < 0.001$.
[2] For cultured lymphocytes, $P < 0.05$; for lymphoma, $P < 0.10$; and for myelocytic leukemia cells, $P < 0.005$.

C/P ratio has been observed in leukemic leukocytes, especially in the most immature blast cells of the acute leukemias (Table 2). The reciprocal alterations in phosphatidylcholine and cholesterol in cultured cells as well as in leukemic cells are further emphasized if expressed as the molar ratio of cholesterol to phosphatidylcholine (C/PC). Normal mature cells had a C/PC ratio of about 1.4, whereas all the leukemic cells studied had values of 1.0 or below, with the lowest values (0.5–0.7) occurring in the acute leukemias. Of the cultured cells, the highest C/PC ratio (0.79) was found in the normal lymphocyte line; the lowest (0.51), in cells derived from myelocytic leukemia.[3]

A high proportion of glycolipid was found in the cultured cells, particularly the Burkitt lymphoma cells. A major portion of the glycolipid fraction migrated on thin-layer chromatograms as a single spot with the mobility of ceramide dihexoside, but smaller amounts of several other glycolipids were detected as well. We have found similar quantities of glycolipid in normal mature lymphocytes and polymorphonuclear leukocytes, consistent with the earlier reports (15, 16) of a high ceramide lactoside content in lipid extracts of mixed populations of normal circulating leukocytes.

DISCUSSION

Comparison of the three lines of cultured leukocytes showed some striking similarities in the lipid patterns of these cell lines, as well as substantial differences from the patterns observed in normal mature cells. As might be expected from the large size of the cells maintained in tissue culture, the total lipid weight per cell was substantially higher than that of circulating normal leukocytes, and the phospholipid content was two to three times that of normal polymorphonuclear leukocytes and lymphocytes, respectively (Table 2). Despite the differences in origin of the parent cells, the cultured cells had nearly identical total phospholipid values and minimal differences in the relative distribution of individual phospholipids. The lipid distribution pattern of these cultured cells was much like the pattern previously observed in leukemic leukocytes, viz., relatively increased phosphatidylcholine, decreased sphingomyelin, and decreased cholesterol in comparison with normal mature leukocytes.

It is unlikely that these changes in lipid composition were a direct effect of the tissue culture medium. Studies of cultured tumor cells and fibroblasts with synthetic media and controlled lipid content (17–19) indicate that the lipid composition of the growth medium readily influences the fatty acid distribution, but not the proportions of individual phospholipids. In analogous in vivo studies with human erythrocytes (20, 21), it has been demonstrated that changes in dietary fat content affect the distribution of red blood cell fatty acids, but not the phospholipid classes.

Cultured Burkitt lymphoma cells had a higher content of triglyceride and glycolipid than the other cell lines maintained in tissue culture (Table 2). Triglyceride accumulation, in the form of intracellular fat droplets, is known to be stimulated in cultured cells by certain changes in the growth medium, such as increased fat content, decreased pH, or agents like phenol and aliphatic polyalcohols (18, 22). In a stable growth medium with carefully maintained conditions, however, other factors must be involved. The Burkitt lymphoma cell culture was known to contain Epstein-Barr (EB) virus, a herpes-type virus. Though little information is available about the specific lipid composition of the EB virus, chemical analyses of viruses generally have shown a lipid pattern that conforms closely to that of the host cell. Some viruses, such as the well-studied influenza virus, contain mostly phospholipid and cholesterol, with very little neutral lipid (23, 24). Other viruses, such as vaccinia, have been reported to contain substantial amounts of triglyceride (25). Virus infection may also lead to altered lipid production by the host (26, 27). A phenomenon of this kind may be responsible for increased amounts of triglyceride and glycolipid observed in the cultured Burkitt lymphoma cells.

A high content of glycolipids was encountered in all three of the cultured cell lines, and the predominant component had the chromatographic mobility of ceramide dihexoside. Ceramide lactoside, first identified as a lipid hapten in epidermoid carcinoma (28), has been found to be the main glycolipid in mixed populations of human leukocytes (15, 16).

In studies of normal leukocytes derived from peripheral blood, the larger and more numerous polymorphonuclear leukocytes in a mixed population of cells contribute most of the lipid. Glycolipid analyses of purified leukocyte preparations[4] indicate that mature lymphocytes as well as polymorphonuclear leukocytes contain substantial amounts of ceramide dihexoside.

Glycolipids appear to be predominantly localized to the cell membrane (29–31). The high degree of species and tissue specificity of this group of lipids and their known capacity to function as lipid haptens (32) indicate that the glycolipids may play an important role in defining he immunochemical specificity of the cell surface. The particularly high ceramide dihexoside content of both mature and immature leukocytes sug-

[3] For cultured lymphocytes and lymphoma, $P < 0.05$; for myelocytic leukemia cells, $P < 0.001$.

[4] Gottfried, E. L., unpublished observations.

gests an especially important function in these cells, which occupy a key position in host immune defenses.

The structural lipid of mammalian cell membranes consists primarily of phospholipids and cholesterol in fixed proportions not only specific for species and cell type (33, 34), but also (at least in some normal mature cells) different in different subcellular organelles (35, 36). It is thus possible that the differences observed between the lipid patterns of normal leukocytes and immature (cultured or leukemic) cells may reflect altered proportions of subcellular membranes, each with its own characteristic lipid distribution. On the other hand, recent studies with blastoid lymphocytes cultured with phytohemagglutinin (37) and rat hepatoma (38) suggest that such undifferentiated cells may develop an undifferentiated membrane pattern with a uniform lipid distribution throughout the cell. This question cannot be answered with certainty until isolated leukocyte plasma membranes are available for lipid analysis.

Surface membranes of malignant and immature cells differ from their normal mature counterparts not only in morphology, but also in functional properties such as electrical surface charge, contact inhibition and adhesiveness to foreign surfaces (39), antigenicity (40), deformability (41), and permeability (42). Indeed, Wallach (43) has suggested that a membrane defect may be the primary factor common to all tumors. In artificial lipid membrane models, even small alterations in the lipid components may produce significant changes in physical and electrical properties (44). Cell membranes, too, may well develop functional changes with alterations in lipid composition. Whether any of the properties of malignant cells are related to the "immature" lipid pattern of high phosphatidylcholine, low sphingomyelin, and low cholesterol content remains to be determined.

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