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Decreased microviscosity of membrane lipids in leukemic cells: Two possible mechanisms

(normal and leukemic cells/plasma membranes and vesicles/serum lipids and lipoproteins)

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ABSTRACT **Steady-state fluorescence polarization studies with the fluorescent lipid probe 1,6-diphenyl 1,3,5-hexatriene were done to determine the degree of microviscosity of cellular membrane lipids and serum lipoproteins in human normal donors and leukemic patients. The results show a marked decrease in microviscosity of cellular membrane lipids in both intact lymphocytes and isolated cellular plasma membranes obtained from leukemic patients in clinical relapse as compared to intact lymphocytes and isolated cellular plasma membranes obtained from normal donors and leukemic patients in complete clinical remission. Concomitant to these dynamic changes in cellular membrane lipids, the degree of microviscosity of lipids in the blood serum of leukemic patients in clinical relapse is markedly reduced as compared to serum obtained from normal donors and leukemic patients in complete clinical remission. Moreover, an *in vitro* incubation of leukemic lymphocytes with normal low density lipoproteins results in an increased microviscosity of cellular membrane lipids. In addition to the interrelation between cellular membrane lipids and serum lipoproteins, plasma membrane vesicles with a high degree of lipid microviscosity were isolated from the blood serum and pleural effusion of leukemic patients in clinical relapse. Such membrane vesicles could not be detected in normal serum. Therefore, we suggest that the two major mechanisms associated with the decreased microviscosity of membrane lipids in human leukemic cells are an abnormal exchange in lipids between the leukemic cell surface membrane and leukemic serum lipoproteins and an exfoliation of plasma membrane vesicles with a high degree of microviscosity from the cell surface of leukemic cells.**

The dynamic nature of a biological lipid complex can be quantitatively monitored by fluorescence polarization analysis with the aid of the fluorescent hydrocarbon probe 1,6-diphenyl 1,3,5-hexatriene (DPH) (1–7). From the recorded degree of fluorescence polarization, the degree of microviscosity of the analyzed sample can be estimated (8, 9). Results obtained with this method have shown that the major parameter that determined the degree of microviscosity of lymphocyte membranes is the molar ratio of cholesterol to phospholipids, the two main lipid components of membranes in mammalian cells (10, 11). In general, the relation between microviscosity and the lipid composition of a given membrane is that an increase in the ratio of cholesterol to phospholipids is associated with an increase in membrane microviscosity and vice versa (10).

Fluorescence polarization studies done in our laboratories have led to the notion that malignancy of lymphocytes, both in experimental animals and humans, is accompanied by a marked decrease in the degree of microviscosity of membrane lipids in leukemic cells as compared to normal lymphocytes (12–16). In accord with results obtained in model systems (9), the main characteristic that determines this dynamic difference originates from a significant decrease in the molar ratio of cholesterol to phospholipids in the plasma membrane lipid core of leukemic lymphocytes (10). Recently, two hypotheses have been advanced to explain these differences between normal and leukemic lymphocytes: exchange of lipids between cellular membranes and serum lipoproteins (1, 17), and exfoliation of plasma membrane vesicles from the leukemic cells with a high ratio of cholesterol to phospholipids (10, 18). Our main interest in the present study was to determine the degree of microviscosity of lymphocyte membrane lipids and of serum lipids in normal donors and leukemic patients and to isolate plasma membrane vesicles from serum of leukemic patients in order to provide experimental evidence for the two hypotheses in human leukemia.

MATERIALS AND METHODS

Normal and Leukemic Lymphocytes. Lymphocytes were isolated from more than 70 blood samples obtained from normal donors and leukemic patients (acute lymphatic leukemia) both in clinical relapse and remission by Ficoll–Hypaque gradient centrifugation (19). The isolated lymphocytes were washed twice with phosphate-buffered saline (P_i/NaCl) and used immediately in the experiments.

Isolation of Cellular Plasma Membranes and Serum Membrane Vesicles. Cellular plasma membranes were isolated from normal and leukemic lymphocytes by a modification of described methods (20, 21). Membrane vesicles (SV) were isolated from blood serum or pleural effusion of leukemic patients in relapse. The degree of purification of plasma membrane and SV fractions was analyzed by determining the specific activity of 5′-nucleotidase (22), glucose-6-phosphatase (23), and acid phosphatase (24). The specific activity of all three enzymes was calculated as μmol of inorganic phosphate (25) released per mg of protein (26) per hr. All plasma membrane and SV fractions were also assayed for total cholesterol (27) and total phospholipids (28). For fluorescence measurements of the isolated plasma membrane and SV fractions, glycerol and sucrose were removed by a 12-hr dialysis against P_i/NaCl.

Serum Lipids, Lipoproteins, and Artificial Liposomes. Fluorescence measurements of normal and leukemic serum were done with 0.1-ml samples of plasma. Low density lipoproteins (LDL) and high density lipoproteins (HDL) were isolated from normal serum as described (29). Mixed liposomes were prepared from egg lecithin and cholesterol. Lecithin (100 mg) and cholesterol (50 mg) were sonicated in 10 ml of P_i/NaCl for 20 min with a Branson sonicator (9).

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Abbreviations: DPH, 1,6-diphenyl 1,3,5-hexatriene; LDL, low density lipoproteins; HDL, high density lipoproteins; SV, serum vesicles; P_i/NaCl, phosphate-buffered saline.

Table 1. Fluorescence polarization and microviscosity at 25° of cellular and serum lipids in normal donors and leukemic patients*

Donor/ patient	No. of samples	Fluorescence polarization		Microviscosity	
		Lymphocytes	Serum	Lymphocytes	Serum
Normal donors	23	0.289 ± 0.007	0.326 ± 0.010	3.91 ± 0.28	5.86 ± 0.65
Leukemic patients in relapse	29	0.250 ± 0.010	0.261 ± 0.020	2.65 ± 0.29	2.98 ± 0.57
Leukemic patients in remission	30	0.287 ± 0.008	0.315 ± 0.010	3.89 ± 0.36	5.27 ± 0.77

* 6×10^6 intact cells and 0.1 ml of plasma (serum) were incubated with 3 ml of DPH/P_i/NaCl for 30 min at 25°. The fluorescence-labeled samples were then subjected to fluorescence polarization analysis at 25°.

Fluorescence Measurements. Lymphocytes (6×10^6), plasma membrane and SV fractions (10–50 μg of protein), and serum (0.1 ml) were labeled with DPH by incubation of the samples for 30 min at 25° with 3 ml of 2 μM DPH dispersed in P_i/NaCl as described (2). The fluorescent, labeled samples were subjected to fluorescence polarization analysis (P) with an Elscimt Microviscosimeter (17). From the recorded P values, the degree of microviscosity ($\overline{\eta}$) of intact cells, isolated membranes, and serum lipids was calculated as described (17). All fluorescence measurements were carried out at 25°.

RESULTS

Results obtained with lymphocytes isolated from the peripheral blood of 23 normal donors, 29 leukemic patients in relapse, and 30 leukemic patients in remission indicated a marked reduction in the degree of fluorescence polarization (P) of lymphocytes isolated from leukemic patients in relapse as compared to lymphocytes isolated from normal donors or leukemic patients in remission (Table 1). These differences in the P values represent a 30% decrease in the degree of microviscosity $\overline{\eta}$ of cellular membrane lipids in the leukemic lymphocytes (Table 1).

In order to establish the extent to which these dynamic differences are related to structural differences in the cell surface membrane lipids, plasma membranes were isolated from normal lymphocytes and from lymphocytes obtained from leukemic patients in relapse. Three membrane fractions were isolated from each cell type according to the degree of enrichment of 5′-nucleotidase (EC 3.1.3.5) over glucose-6-phosphatase (EC 3.1.3.9) and acid phosphatase (EC 3.1.3.2). As with the intact cells, the results showed a marked reduction in the degree of microviscosity of the plasma membranes isolated from leukemic cells as compared to plasma membranes isolated from normal lymphocytes (Table 2). Moreover, lipid analysis of the isolated plasma membranes showed that the ratio of cholesterol to phospholipids was 0.67 and 0.39 for normal and leukemic membranes, respectively (Table 2). Similar results were obtained with three different preparations of plasma membranes isolated from three different normal donors and three different leukemic patients. However, with both cell types (normal and leukemic lymphocytes), the microviscosity of purified plasma membranes was higher than those of the corresponding intact cells, $\overline{\eta} = 3.91$ and 5.42 for intact cells and membranes of normal lymphocytes and $\overline{\eta} = 2.65$ and 3.29 for intact cells and membranes of leukemic lymphocytes (Tables 1 and 2). This may indicate that intracellular membranes also contribute to the DPH fluorescence signals (10); if that is the case, then one can suggest that all cellular membranes, including the cell plasma membrane, have a lower microviscosity in leukemic cells.

An alternative explanation for the differences between intact cells and isolated plasma membranes may be sought in the experimental conditions used for isolation of plasma membranes. During isolation the degree of purification is assayed as an increase in the specific activity of 5′-nucleotidase. If this enzyme is located in the areas of the most rigid plasma membranes, then the areas of fluid plasma membranes that do not contain this enzyme are not taken into consideration. Another possibility is that a specific tensile force that causes stretching of the plasma membrane in intact cells increases the fluidity in its lipid core, as suggested for the erythrocyte membrane (30). To summarize, our present results have shown a 30% decrease in microviscosity of intact leukemic lymphocytes as compared to intact normal lymphocytes and a 40% decrease in microviscosity of isolated leukemic plasma membranes as compared to isolated normal lymphocyte plasma membranes.

Previous studies have indicated that cellular membrane lipids and serum lipids are interrelated parameters in human leukemia (1, 17). Therefore, the degree of microviscosity of lipids was quantitatively monitored in sera obtained from normal donors and leukemic patients. The results (Table 1) showed a marked

Table 2. Enzyme markers, lipid dynamics, and lipid composition in plasma membranes isolated from lymphocytes obtained from normal donors and leukemic patients in relapse

Lymphocytes	Membrane fraction (sucrose density)	5′-Nucleotidase		Glucose-6-phosphatase		Acid phosphatase		Fluorescence polarization*	Microviscosity*	Cholesterol/ phospholipids (mol/mol)*
		S.A.†	E‡	S.A.†	E‡	S.A.†	E‡			
Normal	1.10–1.12	12.1	42	0.46	2.0	1.16	3.6			
	1.12–1.16	3.4	12	0.83	3.7	2.85	9.0	0.321	5.42	0.67
	1.16–1.18	2.8	10	0.24	1.0	0.91	2.8			
Leukemic	1.10–1.12	1.44	24	0.99	3.6	2.13	10			
	1.12–1.16	1.25	20	1.70	6.3	3.12	14.8	0.269	3.29	0.39
	1.16–1.18	0.31	5	1.35	5.0	1.18	5.6			

* Mean at 25°.

† Specific activity of enzymes: μmol of P_i released per mg of protein per hr.

‡ *Enrichment over cell* homogenate. Cell homogenate was centrifuged at 300 × *g* for 10 min. The supernatant was centrifuged at 27,000 × *g* for 20 min. The pellet was then resuspended in 6 ml of sucrose at a density of 1.20 and overlaid with a sucrose gradient (1.08–1.20). The gradient was then centrifuged at 131,000 × *g* for 180 min, and the different membrane fractions were isolated.

Table 3. Increased microviscosity of leukemic lymphocytes induced by incubation in LDL isolated from serum of normal donors and in mixed liposomes of lecithin/cholesterol

Incubation medium	Microviscosity
P_i/NaCl	2.61
HDL	2.57
LDL	3.70
Liposomes*	4.30

Cells (10^7) were incubated in 1 ml of the indicated material for 10 hr at 4°. The concentration of HDL and LDL was as in normal blood serum. After incubation, cells were washed twice with P_i/NaCl and labeled with DPH and the microviscosity was determined at 25°. The data represent the mean values of six independent experiments.
* Ten milligrams of lecithin and 5 mg of cholesterol per ml of P_i/NaCl.

decrease in serum lipid microviscosity of leukemic patients in relapse ($\bar{\eta} = 2.98 \pm 0.57$) as compared to normal serum ($\bar{\eta} = 5.86 \pm 0.65$) or to serum obtained from leukemic patients in remission ($\bar{\eta} = 5.27 \pm 0.77$). Recent experiments in our laboratories have shown that the decrease in microviscosity of leukemic serum is associated with an increase in very low density lipoprotein triglycerides and with a decrease in low density lipoprotein cholesterol (M. C. Glangeaud and M. Inbar, unpublished results). Based on these observations, attempts were made to obtain more direct information regarding the interrelation between membrane lipids and serum lipids, and to determine which fraction in serum is responsible for the degree of microviscosity of cellular membrane lipids. For these experiments, lymphocytes isolated from leukemic patients in relapse were incubated *in vitro* with LDL and with HDL isolated from serum of normal donors. Results showed a marked increase in the degree of microviscosity of leukemic lymphocytes upon incubation in normal LDL, while under the same conditions, no effects were observed with normal HDL. No changes in microviscosity were observed when normal lymphocytes were incubated under the same conditions in normal HDL or LDL. In addition, incubation of leukemic lymphocytes in artificial liposomes prepared from cholesterol and lecithin (mol/mol) (15 mg of lipids per ml) also showed an increase in the degree of microviscosity of the incubated cells (Table 3).

Recent studies with mouse leukemia have suggested that the growth of an ascites tumor is accompanied by formation of plasma membrane vesicles with a high degree of microviscosity that are shed from the cell surface of the leukemic cells into the ascites fluid (10, 18).

In order to determine the extent to which such vesicles are also produced by human leukemic lymphocytes, plasma membrane vesicles were isolated from blood serum and pleural effusion of leukemic patients in relapse. The results (Table 4) showed that in both blood serum and pleural effusion there are membrane vesicles with a high specific activity of 5′-nucleotidase, indicating their plasma membrane origin. Moreover, these plasma membrane vesicles also have a high degree of membrane microviscosity (Table 4). The possibility that these vesicles are formed only by leukemic cells was tested by similar experiments carried out with normal serum. High-speed centrifugation of the same amount of normal serum resulted in a pellet that did not contain 5′-nucleotidase activity. Similar results were obtained with four different normal donors and four different leukemic patients in relapse. These results demonstrated the presence of plasma membrane fractions in serum of leukemic patients in relapse but not in serum of normal donors.

Although the mechanism for formation of these plasma membrane vesicles is not established, we suggest that if they are produced by an active shedding of actively growing leukemic cells, then the specific activity of the plasma membrane enzyme marker, 5′-nucleotidase, should be lower in intact leukemic cells as compared to intact normal lymphocytes. Our present results indeed indicate a marked decrease in this enzyme in the cell surface membrane of intact leukemic lymphocytes; the specific activity (μmol of P_i/mg of protein per hour) of 5′-nucleotidase in intact cells was 0.19 and 0.07 in normal and leukemic lymphocytes, respectively (mean values of 10 different experiments). These results agree with the suggestion that some membrane components, such as membrane cholesterol (10) and 5′-nucleotidase (31, 32), are reduced in the cell surface of leukemic lymphocytes. It is possible that one mechanism is via shedding of plasma membrane vesicles (Fig. 1) with a specific lipid and protein composition (10, 18).

DISCUSSION

Our present observations indicate that the decreased microviscosity of cellular membrane lipids of human leukemic lymphocytes is associated with a marked decrease in microviscosity of serum lipids and the formation of extracellular membrane vesicles with a high degree of membrane lipid microviscosity. These observations suggest that humoral and cellular reactions are the two major parameters that determine the degree of microviscosity of cellular membrane lipids of both normal and leukemic lymphocytes (1, 17). The humoral mechanism may be determined by an abnormal exchange of lipids between the leukemic lymphocyte membranes and an abnormal LDL of leukemic serum. The evidence to support this suggestion was obtained from the experiments indicating a marked decrease in microviscosity of both membrane lipids and serum lipids in lymphocytes and serum obtained from leukemic

Table 4. Enzyme markers and lipid dynamics of membrane vesicles isolated from blood serum and pleural effusion of leukemic patients in relapse

Origin of vesicles	Membrane fraction (sucrose density)	5′-Nucleotidase*	Glucose-6-phosphatase*	Acid phosphatase*	Fluorescence polarization†	Microviscosity†
Blood	1.08–1.18	1.89	0.86	NT	0.293	4.05
serum	1.18–1.20	0.53	0.68	0.92	0.302	4.45
Pleural	1.08–1.18	2.25	0.18	ND	0.320	5.39
effusion	1.18–1.20	5.25	0.16	ND	0.330	6.05

Cell-free blood serum or pleural effusion (130 ml) was centrifuged at 131,000 × *g* for 70 min. The pellet was then resuspended in 10 ml of sucrose at a density of 1.20 and overlaid with a sucrose gradient (1.08–1.20). The gradient was then centrifuged at 131,000 × *g* for 180 min, and the different membrane fractions were isolated. NT, not tested; ND, not detectable.
* Specific activity: μmol of P_i released per mg of protein per hr.
† At 25 °.

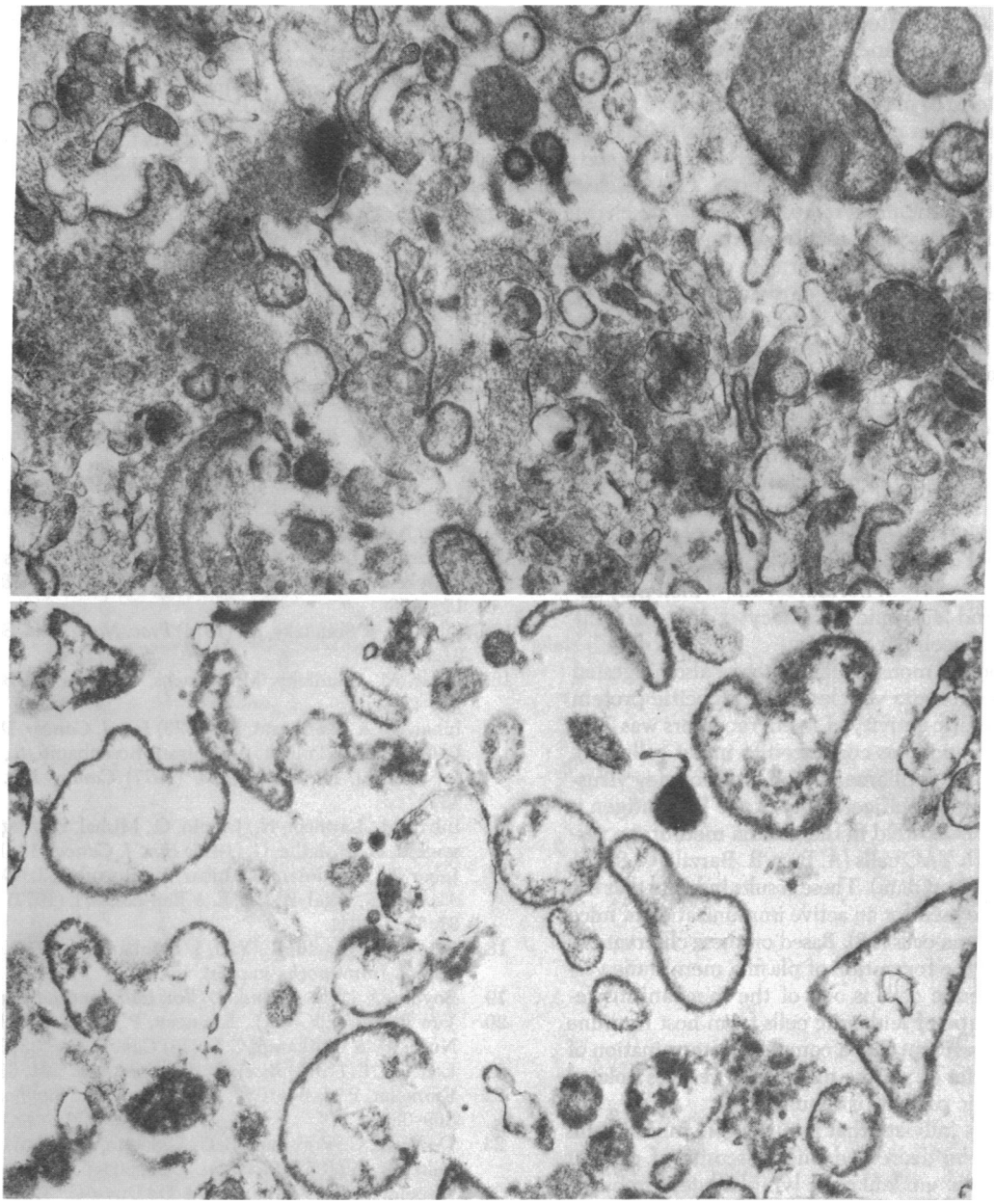

FIG. 1. Electron micrographs of plasma membrane vesicles isolated from pleural effusion of a leukemic patient in relapse before (*Upper*) and after (*Lower*) sucrose gradient isolation. Vesicles were fixed overnight in 2.5% glutaraldehyde in cacodylate buffer, embedded in Epon, and stained with uranyl acetate. Micrographs were taken with a Siemens electron microscope at the magnification of × 26,250. Similar micrographs were also obtained with membrane vesicles isolated from blood serum of leukemic patients in relapse.

patients in relapse as compared to normal donors or leukemic patients in complete clinical remission. It is not clear which changes first, cellular membranes or serum lipids in leukemic patients. However, the fact that leukemic cells can respond to normal LDL or artificial liposomes and can increase their microviscosity, presumably via the translocation of cholesterol from serum lipoproteins (33–35) or liposomes (2, 12) to cellular membranes, may indicate that the ability of the leukemic cell plasma membrane to absorb cholesterol is not affected once this lipid is available (17). According to this notion, a complete analysis of serum lipids of leukemic patients is required. The reduction of serum microviscosity from $\bar{\eta} = 5.86$ or 5.27 in normal donors or leukemic patients in remission to $\bar{\eta} = 2.98$ in leukemic patients in relapse is one of the best indications for a dramatic alteration of the composition of serum lipids (9). Indeed, recent observations (M. C. Glangeaud and M. Inbar, unpublished results) have indicated that two major lipid alterations are responsible for the decrease in microviscosity of leukemic serum: an increase in very low density lipoprotein triglycerides and a decrease in LDL cholesterol. Another possibility is that the degree of exchange of cholesterol between leukemic cells and leukemic LDL is reduced due to a change in the apoprotein of the LDL or in the membrane receptors for LDL. The rapid exchange of cholesterol between normal LDL and leukemic cells may suggest that the membrane receptors are normal; therefore, it will be of interest to determine possible changes in the leukemic LDL apoproteins.

Previous studies with mouse leukemia have shown that the development of a transplantable ascites tumor is accompanied by a significant production of plasma membrane vesicles, which can be isolated from the ascites fluid. Such vesicles were isolated from GRSL leukemia (10), from YAC leukemia (18), and from EL_4 leukemia (unpublished data). These studies have suggested that the formation of these vesicles is associated with a shed-

ding-off mechanism (10, 18). Recent experiments have indicated that these plasma membrane vesicles have a specific lipid composition which can be characterized by an increase in the molar ratio of cholesterol to phospholipids as compared to the isolated plasma membranes of the leukemic cells (10). Our present experiments show a similar situation in both serum and pleural effusion of leukemic patients in relapse. The vesicles isolated from leukemic patients in relapse, which were characterized by electron microscopy, enzyme markers, and lipid microviscosity, have plasma membrane characteristics. Moreover, the degree of microviscosity of these membranes was much higher than that of intact leukemic cells, which may suggest that their formation is one of the mechanisms whereby leukemic cells increase their lipid fluidity (decreased microviscosity) (10).

The possibility that some of these membranes are produced by cell lysis cannot be excluded. However, the fact that these membrane vesicles have plasma membrane enzyme markers of high specific activity and cytoplasmic enzyme markers of very low specific activity is consistent with the idea that the majority are produced by a shedding of viable cells. Such vesicles could not be isolated from normal serum, which may indicate that normal and leukemic lymphocytes differ in their ability to produce extracellular vesicles.

Recent observations on mouse leukemia have also suggested that these plasma membrane vesicles have a specific protein composition. The specific activity of lectin receptors was 3- to 5-fold higher in the vesicles as compared to intact cells (18). Moreover, in the YAC-lymphoma, which is a Moloney virus-induced lymphoma, the specific activity of the P30 antigen is increased by a factor of 20-fold in the plasma membrane vesicles as compared with YAC cells (A. Raz, R. Barzili, G. Spira, and M. Inbar, unpublished data). These results indicate that the isolated vesicles can be used for an active immunization of mice against viable lymphoma cells (18). Based on these observations it was suggested that the formation of plasma membrane vesicles by mouse leukemic cells is one of the mechanisms responsible for the escape of leukemic cells from host immune destruction (18). It is evident that a complete determination of the antigenic properties of plasma membrane vesicles isolated from human leukemic patients is required.

Our present results indicate that two possible mechanisms are associated with the decreased microviscosity of cellular membrane lipids of human leukemic lymphocytes: exchange of lipids between cellular membranes and serum lipoproteins and exfoliation of rigid plasma membrane vesicles from the leukemic cell surface. Although there is no evidence for an interrelation between the two mechanisms, the possibility that an abnormal exchange of lipids between cellular membranes and serum lipoproteins may have a direct effect on the degree of membrane vesiculation cannot be excluded.

In summary, the two associated events that are directly related to the leukemic state—the "fluidization" of cellular membrane lipids via a decrease in the ratio of cholesterol to phospholipids, which may be directly related to the increase in cell proliferation (11), and the formation of extracellular plasma membrane vesicles with a high specific activity of antigens, which may be directly related to the increase in immunosuppression (18)—may determine, to a large extent, the behavior of human leukemia.

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