FLUIDITY OF MEMBRANE LIPIDS: A SINGLE CELL ANALYSIS OF MOUSE NORMAL LYMPHOCYTES AND MALIGNANT LYMPHOMA CELLS

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1. Introduction

Cellular mechanisms which are involved in malignant transformation of normal cells are associated with structural, functional, and dynamic changes in the cell surface membrane [1–6]. Study of these changes, therefore, can be of value in elucidating the molecular basis of normal and abnormal cell growth and development. Among the membrane changes that can be analyzed, dynamic characteristics [7–10], such as, the degree of mobility of membrane protein receptors [11] or the degree of fluidity of membrane lipids [12], can be quantitatively evaluated by analyzing the fluorescence polarization properties of a fluorescent probe which is bound to a specific protein receptor or embedded in the lipid core of the cell surface membrane. Previous studies, carried out in our laboratory, have led to the development of a quantitative technique for the determination of the average membrane fluidity in cell populations [12]. With the aid of this method, we have determined the degree of membrane fluidity of normal and leukemic lymphocyte populations both from experimental animals and humans [13–16]. The results have revealed a marked increase in the fluidity of the surface membrane lipid core of the malignant cells. All these experiments, however, were carried out with cell populations and, therefore, the obtained results were the average value of all the cells present in each population. According to these observations, it becomes increasingly evident that a quantitative analysis of membrane fluidity that can distinguish between normal and malignant cells in a mixed cell population is required. Such an analysis will have a great potential for understanding the control mechanisms associated with the induction and development of the malignant transformed state in the hematopoietic system as well as in other mammalian tissue.

The main issue of the present experiments was to establish a reliable and quantitative method for the determination of membrane fluidity in normal and malignant cells on a single cell level. The method used is based on a single cell analysis of the fluorescence polarization of the fluorescent probe, 1,6-diphenyl 1,3,5-hexatriene (DPH) when embedded in the lipid core of the surface membrane of intact cells.

Abbreviations: SCM, single cell microviscosimeter; DPH, 1,6-diphenyl 1,3,5-hexatriene; PBS, phosphate-buffered saline.

2. Materials and methods

2.1. *Cells*

Normal lymphocytes were obtained from spleen or thymus of A strain mice. The lymphocytes were collected by teasing the tissue apart and allowing the pieces to sediment. The lymphoma cells were from an ascites form of Moloney-virus-induced

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lymphoma (YAC) grown in A strain mice. 10^5 lymphoma cells were inoculated intraperitoneally into adult mice, and the malignant cells were used in the experiments 10 days after inoculation. For each experiment, normal lymphocytes and lymphoma cells were freshly collected from animals in phosphate-buffered saline pH 7.2 (PBS). The cells were used in the experiment after 3 washings with PBS.

2.2. *Liposomes*

Liposomes of lecithin or lecithin–cholesterol (1 : 1, m/m) were prepared as follows: Solutions of 80 mg of egg–lecithin (Lipid Products Ltd., England) or 80 mg of egg–lecithin mixed with 40 mg of cholesterol (Merck, Germany) in 2 : 1 (v/v) chloroform : methanol were evaporated to dryness under nitrogen and dispersed in 10 ml of 0.15 M KCL. The dispersions were then subjected to an ultrasonic irradiation for 30 min with a Branson-130 (Branson, Danbury, Connecticut, USA) at maximum energy output with cooling. The sonicated solutions were then centrifuged at 30 000 *g*, whereupon insoluble material and big liposomes were separated and discarded. Fresh liposomes were prepared for each experiment.

2.3. *Fluorescence labelling of cells and liposomes*

For labelling of cells, 2×10^{-3} DPH in tetrahydrofuran is first diluted 1000-fold with PBS at 25°C yielding a clear aqueous dispersion of 2×10^{-6} M DPH, which is practically void of fluorescence. One volume of cell suspension at the concentration of 10^7 cells per ml is mixed with one volume of DPH dispersion and incubated for 30 min at 37°C. The penetration of DPH into the surface membrane lipid core is followed by a steep increase in fluorescence intensity, which levels off after about 30 min, after which the cells are washed with PBS, resuspended in PBS to a concentration of 10^6 per ml and immediately used in the experiments. For labelling of liposomes with DPH, one volume of liposomes was mixed with one volume of DPH dispersion prepared in 0.15 M KCL and incubated for 30 min at 37°C. Fluidity measurements of labelled cells or liposomes were carried out with the aid of a special fluorescence instrument, the Elscint Single Cell Microviscosimeter (Elscint SCM), which was constructed in our laboratory.

2.4. *Single cell fluorescence polarization analysis*

The instrument which is described schematically in fig.1 is, in essence, a modified fluorescence microscope. Light from a 200 W mercury arc, L, is collimated and passed through a u.v.-transmitting-visible-absorbing filter, F_1, selecting the 360 nm band of the mercury emmission. This light is then polarized horizontally by a Glan-Thompson prism and directed towards the sample, S, by a dichroic mirror, M, passing through the microscope objective, O. The mirror, M, has a high reflectivity in the u.v. and a high transmission in the region of the fluorescence. The fluorescent light is collected by the objective and passed through the mirror, M, to the Wollaston prism, WP, which splits the light into two perpendicular polarized beams, $I_{\parallel}$ and $I_{\perp}$, which are detected by two photomultipliers, PM. A u.v. absorbing filter, F_2, (3 M $NaNO_2$) is used to eliminate any effects of scattered u.v. light and a pinhole, PH, limits the field of view of the photomultipliers to a diameter of 50 μ to ensure that only a single cell is measured at one time.

For the fluorescence polarization measurements of single cells, the DPH labelled cells in PBS were introduced into a haemocytometer chamber which was attached to the microscope stage of the SCM.

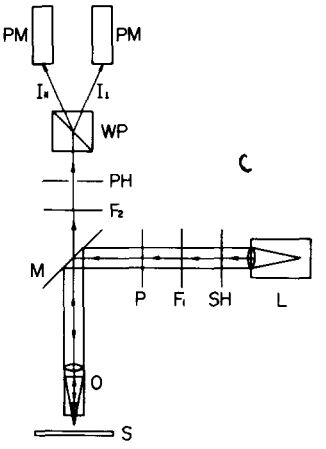

Fig.1. Single Cell Microviscosimeter (SCM). L = 200 W mercury arc; SH = shutter; F_1 = u.v.-transmitting-visible-absorbing filter; P = Glan-Thompson prism; M = dichroic mirror; O = microscope objective; S = analyzed sample; F_2 = u.v. absorbing filter; PH = pinhole; WP = Wollaston prism; $I_{\parallel}$ and $I_{\perp}$ = two polarized beams; PM = photomultiplier.

The measuring procedure involved focusing on the cell to be measured using visible light, with the u.v. shut off by means of the shutter, SH, and then opening the shutter to perform the measurements. This was necessary because of the rapid photobleaching of the DPH. Care was also taken to ensure that the measured cell was outside the illuminated field of all previous measurements with the same hemocytometer chamber. To ensure that each cell is exposed to the same conditions, the fluorescence signals from the photomultiplers were integrated for 200 nsec for each cell after the u.v. light was turned on and then processed electronically to record the value of the degree of fluorescence polarization, P, according to the following equation:

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

In the present experiments, the degree of fluorescence polarization is given in relative units. All measurements were carried out at 25°C. The same set-up was also used for the fluidity measurements of DPH labelled liposomes.

3. Results and discussion

Dynamic parameters of lipid layers were studied extensively during the last decade, mostly with liposomes as membrane model systems [17–19]. These studies have established that three main characteristics determine the degree of fluidity of lipid layers: (a) temperature, (b) the degree of unsaturation of the phospholipid acyl chains, and (c) the mole ratio of cholesterol to phospholipids (C/PL). The most prominent of these characteristics is the C/PL. It was shown that under physiological conditions, at a constant temperature and with naturally-occurring phospholipids, an increase in C/PL will cause a decrease in the fluidity of the membrane lipids [20,21].

In order to test the performance of the Single Cell Microviscosimeter (SCM), and to establish a reference to which fluidity of cellular membranes can be compared, a liposome model system was first analyzed. Experiments were carried out with lecithin liposomes and lecithin–cholesterol mixed liposomes prepared by ultrasonic irradiation and labelled with DPH. The degree of lipid fluidity of the labelled liposomes was determined with the SCM. As displayed in fig.2, the degree of fluorescence polarization of lecithin–cholesterol mixed liposomes was found to be much greater than that of the lecithin liposomes, thus indicating that the lecithin liposomes are much more fluid as compared with the lecithin–cholesterol liposomes. It may be concluded, therefore, that increasing the value of C/PL from 0 to 1 markedly decreases the fluidity of the lipid layers, as might be expected.

In parallel to the liposomes experiments, intact normal lymphocytes and lymphoma cells labelled with DPH were also measured with the SCM. Previous studies have presented a series of indirect evidence suggesting that in DPH labelled intact cells, the fluorescence probe is located in the cell surface membrane [16]. This notion is further supported by a more recent study in which we summarized the direct evidence which clearly shows that the fluorescence signals of DPH labelled intact cells are predominantly obtained from DPH molecules located only in the cell surface membrane lipid core. The

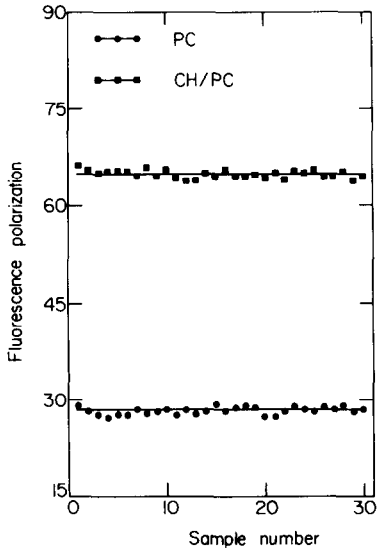

Fig.2. Fluorescence polarization of DPH labelled liposomes. PC = lecithin liposomes (8 mg egg–lecithin/ml); CH/PC = cholesterol–lecithin mixed liposomes (4 mg cholesterol and 8 mg egg–lecithin/ml). Liposomes were prepared by ultrasonic irradiation, labelled with DPH and measured at 25°C.

conclusive evidence for this was obtained by the study in which the degree of fluorescence polarization of DPH embedded in intact cells and in their isolated plasma membranes have shown similar values. These recent studies were carried out in parallel with normal lymphocytes and with leukemic cells (Inbar et al., submitted for publication). Based on these observations, we can assume that with intact cells labelled with DPH, the major contribution to the fluorescence signal originated from the surface membrane and, thus, this method can serve as a quantitative tool for the study of dynamic parameters of the surface membrane lipid layer in its intact structure.

Previous studies [12–15] with normal and leukemic populations from both experimental animals and humans have established the observation that the surface membranes of the leukemic cells have a more fluid lipid core than that of normal lymphocytes. The increase in fluidity in the leukemic cells is predominantly caused by a decrease of C/PL in the cell surface membrane [15] and in human leukemia it is in correlation with the acuteness of the disease (Inbar and Ben-Bassat, submitted for publication). Moreover, in studies in which attempts have been made to elucidate the biological significance of membrane fluidity, it was shown that the reduction of membrane fluidity induced by the introduction of exogeneous cholesterol into the surface membrane of intact lymphoma cells from mice resulted in a marked inhibition of their tumorigenicity [14]. On the other hand, a controlled increase in membrane fluidity of normal lymphocytes induced by a reduction of membrane cholesterol resulted in a significant increase in the activation of the normal lymphocytes by plant mitogens (Inbar, in preparation).

For the determination of membrane fluidity in intact cells, normal lymphocytes and lymphoma cells freshly isolated from mice were first labelled with DPH and the degree of fluorescence polarization on a single cell level was monitored with the aid of the SCM. Fig.3 presents the distribution of membrane fluidity in cells from the normal cell population versus cells from the malignant cell population. No significant difference in results between normal lymphocytes obtained from mouse spleen or thymus was observed. As shown, the leukemic cell population approaches an upper limit value of fluorescence polarization which is close to the lower limit value of the normal population, thus indicating that the malignant lymphoma cells have a more fluid membrane as compared with the normal lymphocytes. The results clearly demonstrate that the degree of fluorescence polarization of DPH embedded in the surface membrane lipid core can serve as a quantitative tool to distinguish between normal and leukemic cells on a level of one single cell.

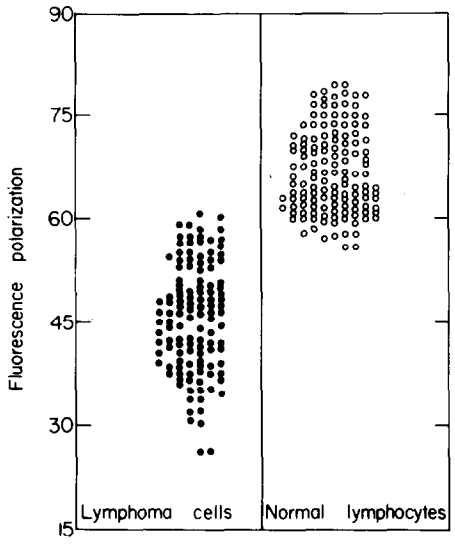

Fig.3. Fluorescence polarization of DPH-labelled normal lymphocytes and lymphoma cells. Cells were freshly obtained from A strain mice, labelled with DPH and measured at 25°C. Each symbol represents one single cell.

Liposomes can exchange sterols with cell surface membranes [12] in a fashion similar to lipoproteins [22]. Presumably, the exchange mechanism involves a physical contact between the liposomes and the exposed lipid regions on the membrane, through which the sterols are translocated according to their partition characteristics [15]. Introduction of exogeneous cholesterol into the surface membrane of intact cells can be performed with lecithin–cholesterol (m/m) liposomes (12 mg lipid/ml) [12]: in the malignant lymphoma cells it resulted in an increase in the degree of fluorescence polarization of DPH (decrease in membrane fluidity) (Fig.4). Extraction of native cholesterol from the surface membrane can be performed with lecithin liposomes

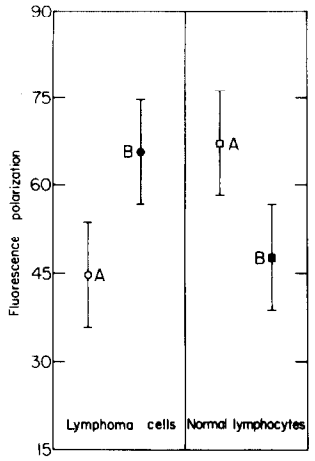

Fig.4. Fluorescence polarization of lymphoma cells and normal lymphocytes with and without treatment with liposomes. Left – lymphoma cells – (A) untreated cells, (B) cells treated with CH/PC liposomes (12 mg lipid/ml). Cells were incubated for 2 hours with liposomes at 4°C then washed with PBS and labelled with DPH. Right – normal lymphocytes – (A) untreated cells, (B) cells treated with PC liposomes (8 mg lipid/ml). Cells were incubated for 2 h with liposomes at 4°C then washed with PBS and labelled with DPH. All measurements were carried out with the SCM at 25°C. For each experiment cells were freshly isolated from A strain mice.

(8 mg lipid/ml) [12]: in normal lymphocytes it resulted in a decrease in the degree of fluorescence polarization (increase in membrane fluidity) (Fig.4). These results, which are in line with our previous studies, have clearly supported by notion that the difference in membrane fluidity between normal and malignant lymphocytes mainly originated from the difference in the C/PL mole ratio in the cell surface membrane of the two cell types [15].

Our previous and present studies have indicated that fluorescent molecules embedded in the surface membrane lipid core of intact cells can be used to elucidate dynamic structural changes of membrane components in relation to those cellular regulatory mechanisms associated with malignant transformation of normal cells. By analyzing the fluorescence polarization properties of the fluorescence probe, DPH, when embedded in membrane lipids, it was shown that there is a direct correlation between the degree of lipid fluidity in the cell surface membrane and the malignantly transformed state of cells from the hematopoietic system. In both experimental animals and humans, leukemia is accompanied by an increase in the membrane fluidity of the leukemic cells, and, therefore, differences in membrane fluidity, when determined on a single cell level, can be of value for the distinction between hematopoietic cells of normal and neoplastic origin. An extension of our present studies on fluidity analysis of single cells to other mammalian tissues can provide an effective approach to the study of basic differences between normal, premalignant and malignant cells.

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