FLUORESCENCE POLARIZATION OF 1,6-DIPHENYL-1,3,5-HEXATRIENE EMBEDDED IN MEMBRANES OF MOUSE LEUKEMIC L 1210 CELLS DURING THE CELL CYCLE

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1. Introduction

Amongst the numerous events which occur during a cell cycle, several are localized at the level of cell surface membrane. By labelling normal and transformed cells with fluorescent lectins, changes occurring during the cell cycle have been demonstrated [1–3]. With normal cells, the binding of lectins increased during mitosis and with transformed cells it increased during interphase. Furthermore, intramembranous particles were aggregated during several phases of the cell cycle but not during the mitotic phase [4]. More recently, it was suggested that the fluidity of normal and transformed mammalian fibroblasts in culture decreased when the cell density increased [5,6] and that the fluidity of various mammalian cells increased [7] when cells were stimulated for growth. All these results suggest that the cell membrane undergoes a dynamic change during different phases of cell cycle.

In the present report, murine leukemic cells L 1210 were synchronized with colcemid and the expected changes were checked by fluorescence polarization measurements of 1,6-diphenyl-1,3,5-hexatriene [8] embedded in lipid bilayer. These results are discussed on the basis of fluorescence decay studies of DPH incorporated in unsynchronized L 1210 cells [21].

2. Materials and methods

2.1. *Cell culture*

The cell line used throughout this study was L 1210 mouse leukemic cells adapted to grow in suspension culture. The cells were grown at 37°C in a humid atmosphere at 95% air and 5% CO_2 in a water-jacketed CO_2 incubator (National Appliance, a Heinke Company). The medium employed was Eagle's minimum essential medium (MEM). The cell density never exceeded 1×10^6 cells /ml.

2.2. *Synchronization of L 1210 cells*

Synchronized cells were prepared from exponentially growing cells (5×10^5 cells/ml) by addition of colcemid (0.06 μg/ml) in culture medium [9]. After 12 h, cells were centrifuged at $1200 \times g$ for 10 min, at 37°C. The supernatant was poured off and the cells were then suspended in fresh sterile medium.

The progress of the cell cycle was determined on portions of the synchronized culture. These portions were maintained at 37°C in the presence of 5% CO_2 in fresh MEM medium; cells were then harvested at various time intervals.

2.3. *Incorporation of [^{3}H]thymidine*

At 1.5 h intervals after synchronization 1 ml cells in suspension was pulse labelled (20 min) at 37°C with 20 μl [^{3}H]thymidine at 0.1 μCi (CEA-France, 25 Ci/mM). The reaction was stopped by adding 2 ml cold (PBS) containing 0.6 mM thymidine and centrifugation 10 min at $1800 \times g$. The cells were washed a second time with the same solution, and ^{3}H-labelled cells in suspension (1 ml) were added to scintisol complete solvent (10 ml) (Scintix) and counted in a Beckman Tri-Carb liquid scintillation counter.

2.4. *Determination of mitotic cells [10]*

Cells in 1 ml culture medium were treated with

2 ml hypotonic solution, 0.075 M KCl, for 10 min at 37°C and fixed with acetic acid–methanol solution (1:3). A few drops of the cell suspension in the fixative were spread on a clean slide, air dried and stained with Giemsa solution. The incidence of metaphase cells was determined by examining more than 500 cells with a light microscope.

2.5. *Fluorescent labelling of cells [8]*

A stock solution of 1,6-diphenyl-1,3,5-hexatriene (Koch Light Ltd, England) (2×10^{-3} M) in tetrahydrofuran was prepared. For labelling the cells, this solution was first diluted 1000-fold in PBS, pH 7.4, with vigorous stirring. Stirring was continued for 15 min at 25°C and 1 vol. clear solution was added to 1 vol. cell suspension (1×10^6 cells/ml). To allow complete DPH incorporation, the suspension was incubated 30 min at 25°C with gentle shaking. The cell suspension was then centrifuged (2000 rev/min, 10 min) and resuspended in PBS. The final concentration was 5×10^5 cells/ml. The labelled cells were immediately used for fluorescence measurements.

2.6 *Fluorescence measurements*

Fluorescence measurements were performed with an Elscint MV-1 fluoropolarimeter [11,12]. This instrument is equipped with a 100 W mercury arc, a 366 nm excitation filter, Glan-Thompson polarizers, 2 emission cut-off filters for wavelengths below 390 nm and a constant temperature chamber.

The fluoropolarimeter simultaneously analyzed the fluorescence intensities detected through two polarizers oriented parallel ($I_{\parallel}$) and perpendicularly ($I_{\perp}$), respectively, to the direction of the polarized excitation beam. The fluorescence polarization P was calculated according to the formula:

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

with an accuracy of ± 0.003.

3. Results

3.1. *Fluorescence polarization of DPH in unsynchronized cells*

Relative to normal mouse lymphocytes, L 1210 cells, like all leukemic cells, exhibited a very low fluorescence polarization *(P)* of DPH, expressing a high fluidity of the membrane. Different values of P, observed from one seeding to another one, indicated a heterogeneity of their population and the influence of the serum batches (table 1).

Table 1
P values at 25°C and 37°C of unsynchronized L 1210 cells labelled with DPH ($P \pm 0.03$)

Exp.	25°C	37°C
1	0.179	0.138
2	0.160	0.120
3	0.140	0.112
4	0.157	0.126
5	0.159	0.127

When DPH was added to a suspension of washed cells, the fluorescence intensity of DPH increased rapidly and reached a plateau within 30 min. The P values were higher during the first minutes of incubation but decreased rapidly and remained constant after 15 min, and were not dependent upon the cell concentration in the range of 5×10^5 to 2×10^6 cells/ml. The absence of change related to the cell concentration showed that light-scattering depolarization could be neglected. The fluorescence polarization of DPH was shown to decrease monotonically when temperature increased from 5–37°C (fig.1).

3.2. *Cell synchronization*

When synchronized cells were suspended in fresh culture medium, all cells complete their division

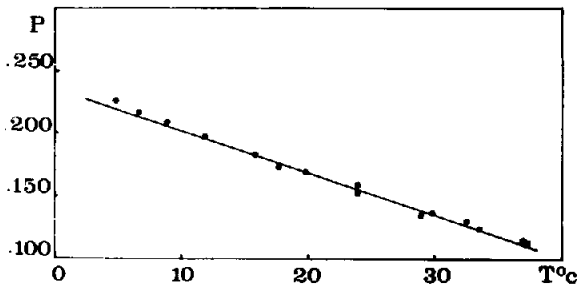

Fig.1. Degree of fluorescence polarization of DPH-labelled cells related to temperature from 5–37°C. (•) Decreasing temperature. (▪) Increasing temperature.

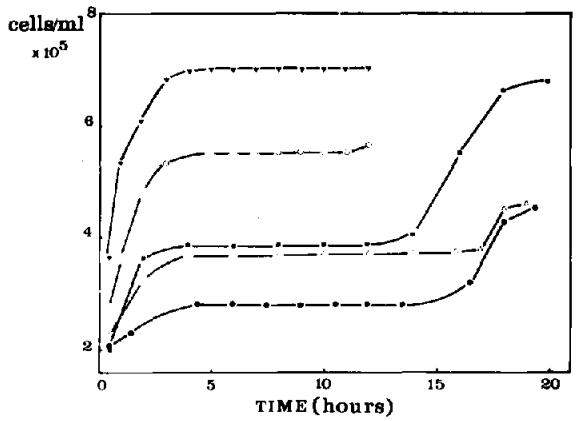

Fig.2. Number of cells related to the time after colcemid removal.

within 3–4 h; during this interval, the number of cells increased 2-fold (fig.2) and then remained constant for 11–12 h. The overall durations of the cell cycle were found to be constant (14–16 h) in all experiments.

The mitotic index of the populations used ranged from 21–41% (table 2) indicating a variation in the response of leukemic cells to the synchronization agent. In most cases, the synchronization was found to be quite high (35–40%). In control experiments with unsynchronized cells in the exponential phase, the mitotic index varied from 0.5–1%.

Table 2
Cell synchronization data of 8 independent experiments

Exp.	a (h)	b (p 100)	c (h)	d (max/min)
1	4	35	7	2.3
2	–	21	10	2.5
3	2	31	–	–
4	4	41	10	3.0
5	4	43	13	8.5
6	–	38	6	1.8
7	4	24	6	5.3
8	3	31	12	3.9

[a] Duration of the division phase
[b] Mitotic index, p 100
[c] Time of the maximum uptake of [^{3}H]TdR after removal of colcemid
[d] Maximum to minimum ratio of [^{3}H]TdR uptake

The ratio of the maximum and of the minimum of [^{3}H]TdR incorporation changed from 2.3–8.5 (table 2). However, these variations could not be directly related to the degree of synchronization, as estimated by the mitotic index.

The time between the removal of colcemid and the maximum uptake of [^{3}H]TdR, varied from 6–13 h. Amongst all the phases of the cell cycle, G_1-phase was the most variable, and S-phase the most constant.

3.3. *Degree of fluorescence polarization*

In each experiment, the variation of P values (fig.3) throughout the different phases of the cell cycle never exceeded the variation usually observed in the experiments with unsynchronized cells. In most cases, the P values of unsynchronized cells were slightly higher than those of synchronized cells.

4. Discussion

According to the high mitotic index, the use of colcemid to block cells in metaphase was found to be quite suitable to synchronize L 1210 cells. Throughout all our experiments and in spite of the relatively long colcemid treatment (12 h), L 1210 were all alive.

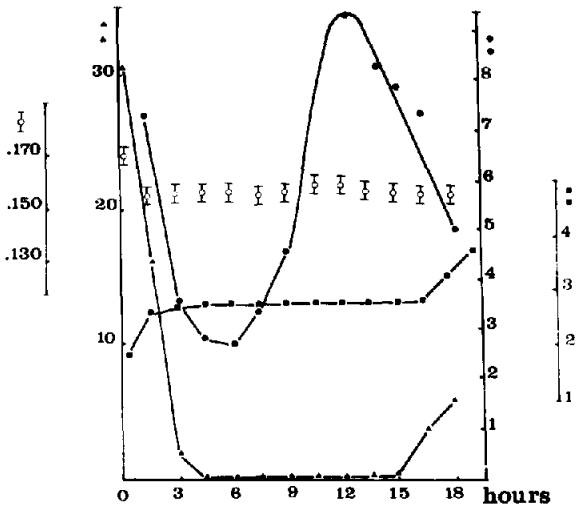

Fig.3. Synchronization experiments. Degree of fluorescence polarization *(P)* at 25°C (Ŧ), the bar represents the average of experimental variations. (▲) Mitotic index. (●) Uptake of [^{3}H]TdR, cpm/10^5 cells. (■) Number of cells during a complete cell cycle.

With unsynchronized cultures, the uptake of [^{3}H] TdR is often identical to or sometimes greater than the maximum uptake by synchronized cells. This behaviour may be related to the very different response of unsynchronized and synchronized cells, towards addition of thymidine, with respect to the enzymes involved in the synthesis of DNA [13,14]. We have also to remember that colcemid treatment disturbs the assembly of microtubules and induces changes in the capacity of cells to move [15–17]; therefore even after colcemid has been removed from the medium, cells are partially affected by the previous colcemid treatment. In a growing culture, the duration of G_1 differs by up to fourfold from one cell to another, indicating a loose coupling in the interval between division and DNA synthesis [12]. All cells may go into a quiescent state after division [19] and the probability of escaping from this state may be typical of each cell. Some biochemical processes, which are not important to cell division, are involved in this G_1-phase [20]. On the contrary, the S-phase, which involves some distinct limited functions required for cell division, e.g., DNA synthesis and the preparation for mitosis was very constant (6–7 h).

In all experiments with synchronized cells, P was found to be quite constant through the whole cell cycle. The fluorescence polarization is an average measurement which depends of several parameters: behaviour of probe molecules in the bilayers, and structure and properties of their surroundings. Constant values of the fluorescence polarization of DPH in L 1210 synchronized cells may be due to one of several explanations:

(i) The DPH is located in plasma membranes as well as in other intracellular membranes [24,25]; it is possible that the variations of the fluidity of L 1210 cell surface membrane are masked because of the lack of sensitivity of this technique.

(ii) The heterogenous environment of DPH in L 1210 cells [21] as in liposomes [22,23] can provide a constant value related to a combination of opposite effects. The DPH molecules embedded in L 1210 membranes were shown to have at least 2 distinct distributions: 58% molecules with a lifetime of 9 ns and 42% with a lifetime of 4.5 ns. Furthermore, the emission, anisotropy of DPH embedded in L 1210 cells did not follow a single exponential decay but could be resolved into two phases: an initial fast decreasing phase; and a second almost constant phase. This finding can be interpreted in terms of microheterogeneity of sites for the probe or in term of DPH rotation anisotropy in the bilayer. This implies that the interpretation of steady state data in term of 'microviscosity' is not reasonable and lead us to express all our results as values of P, indicating an average motion of the probe.

(iii) Although the variations of the fluidity at different phases of the cell cycle are observed for some normal synchronized cells [7,26], it is possible that the fluidity of transformed cells, such as leukemic L 1210 cells, remains constant throughout all phases.

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