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LATERAL DIFFUSION OF MEMBRANE D-GALACTOSYL GLYCOCONJUGATES OF DIFFERENTIATING NEUROBLASTOMA CELLS

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SUMMARY : Neuroblastoma cells were induced to differentiate either by serum deprivation or addition of dimethylsulfoxide. Using the "fluorescence recovery after photobleaching method" (FRAP), the lateral diffusion properties of D-galactosyl glycoconjugates revealed with fluorescent labelled peanut agglutinin (PNA) was investigated. Statistical significant modifications were found on process-bearing cells only for the characteristic diffusion time. The mobile fractions of PNA binding sites were distributed about a mean value of 60 %. Attempt was made to discuss the FRAP results in term of cell-to-cell variation.

INTRODUCTION

Murine neuroblastoma cells have been widely used as a model system for neuronal cells as they can be induced to "differentiate" in culture by various stimuli such as serum starvation (1) or dimethylsulfoxide (DMSO) (2). Although direct analogies between normal neurons and "differentiated" neuroblastoma cells should be made with caution (3), neuroblastoma cells express several neuronal characteristics including neurotransmitter synthesis (4), electrical activity (2) and process formation (1, 2, 4). Moreover, alterations in cell-membrane properties such as surface glycopeptide (5) or lipid composition (6) and lateral diffusion of membrane components (7, 8) were reported as being associated with the differentiated state.

Neuroblastoma cells, clone NS20 (4), are known to interact with concanavalin A and ricin (7, 9). Recently, we have demonstrated that these cells possessed peanut agglutinin (PNA) receptors sites, even when induced to differentiate (10). This lectin is specific for terminal D-Galactosyl residues (11) and in other model systems, the acquisition or disappearance of such residues seem to be linked to an in vivo and in vitro differentiation (12, 13).

Abbreviations : PNA, peanut agglutinin ; TRITC, tetramethylrhodamine isothiocyanate ; DMSO, dimethylsulfoxide ; FRAP, fluorescence recovery after photobleaching.

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The aim of this investigation was to study the modulation of PNA receptors lateral diffusion in relation with the morphological differentiation of NS20 neuroblastoma cells. We have used the "fluorescence recovery after photobleaching" (FRAP) method which allows measurements on single living cells (14). In addition, we shall discuss the FRAP result variations, also mentionned in other works (15, 16, 17, 18).

MATERIALS AND METHODS

Cells . C 1300 mouse neuroblastoma cells, clone NS20, were grown on glass coverslips in Eagle's minimal essential medium containing 10 % fœtal calf serum (Eurobio), glutamine (2 mM – Eurobio) and penicillin – streptomycin (100 IU/ml – 100 mg/ml). Cells were replated in 35 mm plastic petri dishes at 1 - 2 x 10^5 per dish. Dishes were incubated in an atmosphere of 5 % CO_2 - 95 % air at 37°C. Differentiation was induced 48 h after plating either by absence of fœtal calf serum (1) or by addition of 2 % (vol/vol) dimethylsulfoxide (DMSO) in the growth medium (2).

Fluorescent PNA . PNA was prepared by affinity chromatography on desialylated polymerized human red cell ghosts (19, 20). To conjugate the lectin with rhodamine derivative (TRITC isomer R - BBL), a method close to that reported for immunoglobulin was used (10). The absorbance ratios of the conjugate, OD550/OD280 and OD515/OD550, were respectively 0.25 and 0.9. When native and fluorescent PNA were compared, their hemagglutination activities, reactivities against anti-native PNA antibodies and electrophoretic mobilities in polyacrylamide gradient gel were not found to be significantly modified (10).

Fluorescent labelling . Cells were rinsed 3 times with Hank's balanced salt solution (HBSS) and then incubated with 100 µg of PNA-TRITC in 1 ml of phosphate buffer solution (PBS). The labelling was performed for 30 minutes at room temperature in the dark and stopped by washing several times with HBSS. For each separate culture dish the same conjugate PNA-TRITC was used. A very low fluorescent signal was detected when galactose (0.05 M) saturated PNA-TRITC was used for cell labelling.

Lateral diffusion measurement . Diffusion coefficients of the fluorescent labelled PNA receptors were determined by the "fluorescence recovery after photobleaching" (FRAP) method (14). Fluorophores within a small area were irreversibly photobleached by a short pulse (2 sec) of intense focused laser light (Argon ion laser - Spectraphysics - 2 mW - λ = 514.5 mn). The laser beam was focused through the microscope (Leitz - MPV2) objective (x 125) to a small spot with 1.75 µm e^{-2} radius. No adjustments of the laser beam were performed during the period of experiments.
Rates of diffusion were determined from the recovery of the fluorescence of unbleached fluorophores from adjacent parts of the membrane into the bleached region (14). To avoid unwanted photobleaching during the measurement, short pulses of continuous attenuated laser light (120 µW) were generated by means of an accousto-optical shutter device (SORO Optics). Duration (200 msec) and frequency (0.5 or 0.2 Hz) of the pulses were monitored by a computer (PDP 11/34) which also calculated the average value of the fluorescence signal detected by a photomultiplier (EMI 9558). Parameters of the measurement and values of fluorescence intensity versus time were stored in a file. Collected data were edited on a line printer and the recovery curves plotted on an X-Y recorder.

Data analysis . Routinely, the Fo/Fi ratio (post-bleach level/pre-bleach level of fluorescence) was calculated, using the data collected by the computer. In our experimental conditions, this ratio was 50 $\pm$ 10 %. The experimental half-recovery time T1/2, deduced from the FRAP curves, was then corrected, according to Axelrod's theory

(14). This treatment allowed the use of characteristic diffusion time T_D (i.e the corrected T1/2) as a comparative measure of apparent mobility (15, 16). Degree of recovery of bleached fluorescence, denoted as R, was directly obtained from the FRAP data.
In order to compare the mean values of T_D and R between two groups of experiments, a modified Student's t test (21) was applied. Because of cell-to-cell variations of FRAP results, linear least square regression studies (21) were in some cases realized.

RESULTS AND DISCUSSION

Neuroblastoma cells, clone NS20, were induced to differentiate either by serum deprivation or addition of DMSO. In agreement with previous works (1, 2), cells in both conditioned medium developed important neurites whereas in growth medium most of them were round.

In order to investigate the changes of the dynamic properties of membrane components, as associated with the differentiated state of the cells, we evaluated the lateral diffusion of PNA receptors by the "fluorescence recovery after photobleaching" (FRAP) technique (14). All measurements were performed on cell somas. The FRAP curves we obtained were similar to data previously reported for a single diffusion coefficient (22). Controls were done to see whether the values of the characteristic diffusion time T_D derived from two successive bleaches of the same membrane location were not significantly different (see fig. 1). In addition, incomplete recovery after the 2nd bleaching was always observed. Given the relative error done on the calculation of the degree of recovery, we found that the apparent fraction of mobile sites after the 2nd bleaching was sometimes equal (as shown on fig. 1) and sometimes higher than the mobile fraction determined after the 1st illumination. In the case of kinetics with several bleachings and incomplete recovery, it seems important not to neglect the possibility that mobility of receptors may occur within limited areas (9).

The data are given in table 1. Table 2 shows the conclusions of Student's tests. It appears that about 55-60 % of the PNA receptors are mobile on the experimental time scale, whatever the culture conditions of neuroblastoma cells. Significant changes are found only for the T_D values. The minimal T_D (i.e. the maximal diffusion coefficients) are obtained for cells in conditioned medium (2 days without serum and 5 days in presence of DMSO). These cultures were characterized by cells which had developed neurites. These

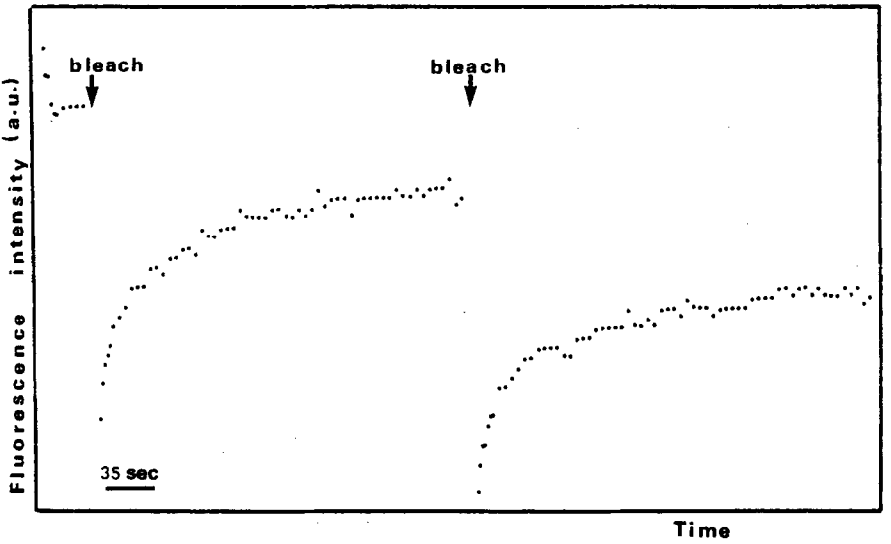

Figure 1 : Photobleaching recovery curve of a successively bleached spot on a PNA-TRITC labelled neuroblastoma cell. After the 1st bleaching : T_D = 18.0 sec, R = 70 %. After the 2nd bleaching : T_D = 18.4 sec, R = 64 %. Maximal relative error done on the calculation of the degree of recovery R was estimated to be 10 % of the experimental value.

results confirm our previous experiments on Concanavalin A (7) and are consistent with works of de Laat et al. (8, 17). However FRAP experiments often give insufficient data to verify that measurements are normally distributed about mean values as required for the

Table 1 : Characteristic diffusion times (T_D) and PNA receptors mobile fractions (R) of neuroblastoma cells under different culture conditions. The results are given as mean $\pm$ S.D. N is the number of independant measurements. The order of magnitude of the diffusion coefficients D ($D = w^2/4\ T_D$, w is the radius of the laser beam) is 10^{-10} cm^2/sec.

Neuroblastoma cells	Lateral diffusion of PNA receptors		
	T_D (sec)	R (%)	N
Growth medium			
5 hours	16.5 (4.2)	58 (12)	12
2 days	15.8 (5.3)	55 (18)	9
Conditioned medium			
Without serum – 2 days	10.7 (3.1)	55 (9)	10
With 2 % DMSO – 2 days	14.1 (6.7)	58 (11)	13
With 2 % DMSO – 5 days	10.7 (4.7)	59 (12)	12

Table 2: Statistical t tests applied on the different groups of FRAP measurements (cf. table 1). + means that the probability that difference is statistical only is less than 0.05.

	With serum (5 hours)	With serum (2 days)	Without serum (2 days)	With DMSO (2 days)	With DMSO (5 days)
With serum (5 hours)		−	+	−	+
With serum (2 days)	−		+	−	+
Without serum (2 days)	−	−		−	−
With DMSO (2 days)	−	−	−		−
With DMSO (5 days)	−	−	−	−	

R values

T_D values

statistical analysis (21). Moreover, large standard deviations of both diffusion variables (see table 1) are common to FRAP measurements. These uncertainties are refered to as cell-to-cell variations by several authors (15, 16, 17, 18).

It can be argued that cell-to-cell variations are due i) to the extent of the FRAP experiments (about 60-90 minutes per sample) ii) to difference between fluorescence intensities of each measured cell location. A control showed that the two FRAP variables were independent of the duration of the experiment (coefficients of determination $r < 0,5$). The second argument can also be eliminated by a linear least square regression test, as illustrated on fig. 2. These cell-to-cell variations might then be due to different positions within the cell cycle. This assumption, suggested by the results of (17), is also supported by our experimental data. Variations are indeed markedly reduced for the serum deprivated neuroblastoma cells (see fig 2) which have been reported to be synchronized to some extent in G1 phase (1).

It can generally be assumed that both diffusion variables, T_D and R, depend on various elements which characterize a given cell state (or a given cell location state). We therefore sought to determine in what way R and T_D were concommitantly modified. A R versus T_D plot (fig. 3) shows that 70 % of our experimental R values are grouped about

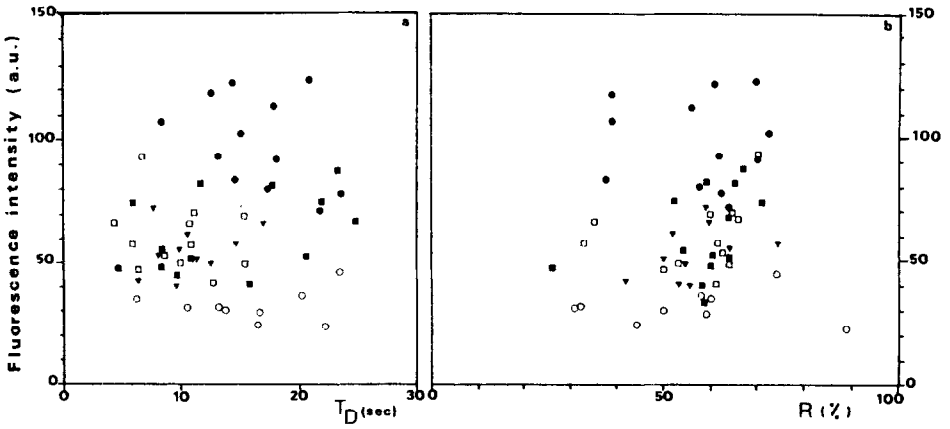

Figure 2 : Fluorescence intensities (a.u. = arbitrary units) of each measured cell location versus a) T_D (sec) b) R (%). Different symbols represent different FRAP experiments on PNA-TRITC labelled neuroblastoma cells : after 5 hours and 2 days in growth medium (● and ○), after 2 days without serum (▼), after 2 and 5 days with 2 % DMSO (■ and □). For the five groups of experiments, coefficients of determination r calculated from both plots a) and b) are less than 0.4.

an average of 60 %, when T_D is distributed from 6 sec. to 25 sec. Within these limits, the fraction of mobile sites does not seem to influence the characteristic diffusion time of PNA receptors.

These results suggest that the induction of neuroblastoma cells differentiation leads only to modifications of cellular elements which influence the characteristic diffusion time of PNA receptors, without alteration of the quite stable mobile fraction. A direct chemical implication of the inducer itself, especially DMSO (23), can be excluded since analogous effect is produced by serum starvation. We may gather from the analysis of cell-to-cell variations that position within the cell cycle is more involved than morphological differentiation in the increase of the diffusion coefficient of PNA receptors on process - bearing cells. It is known that extension of neurites is associated with important cytoskeleton development (24) which contributes in some systems to the control of membrane protein mobility (25). Whether or not "differentiation" of neuroblastoma cells is associated with modifications of the cytoskeleton-membrane interactions is still to be determined. Furthermore, as suggested by a very recent work (26), PNA binding sites may not be linked to the cytoskeletal network.

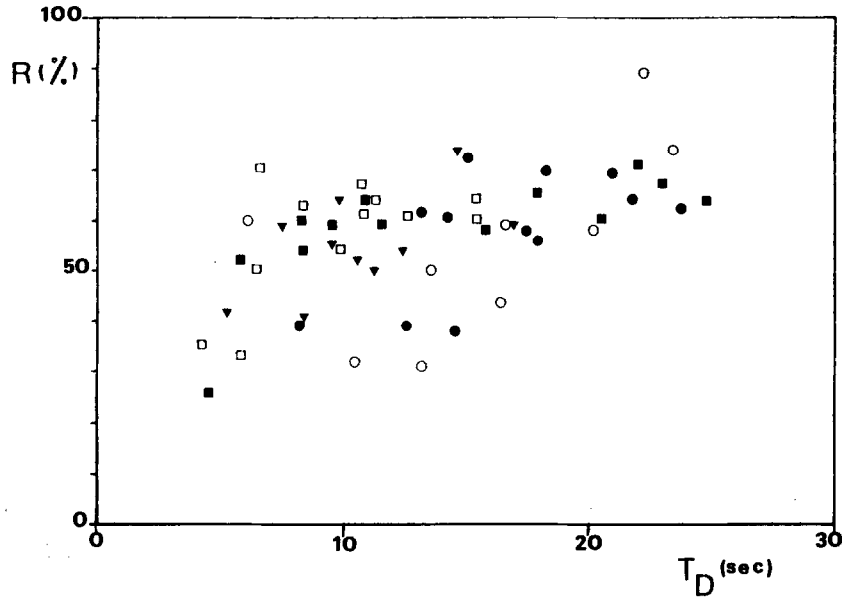

Figure 3: Percentage of PNA mobile receptors as a function of their characteristic diffusion time. Each point represent one measurement on one cell. The symbols are identical to those used in figure 3.
Note that: $R < 50\ \%$, $4\ \text{sec} \leqslant T_D \leqslant 16\ \text{sec}$ (11 values); $50\ \% \leqslant R \leqslant 70\ \%$, $6\ \text{sec} \leqslant T_D \leqslant 25\ \text{sec}$ (40 values) and $R > 70\ \%$, $15\ \text{sec} \leqslant T_D \leqslant 24\ \text{sec}$ (5 values)

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