DIFFERENCES IN MEMBRANE STRUCTURE BETWEEN SUSPENDED AND ATTACHED MOUSE NEUROBLASTOMA CELLS

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1. Introduction

Fluorescence spectroscopy offers a way to measure biophysical parameters of the membranes of living cells by the use of fluorescent probes [1,2]. The probe is bound to membrane sites by electrostatic and hydrophobic forces, usually with a substantial increase in fluorescence yield and a shift of the emission maximum toward shorter wavelengths [3,4]. Measurements of this kind can easily be made on a cell suspension with a normal spectrofluorimeter. However, there is evidence that suspension-cultured cells will change their membrane structure upon attachment to a surface [5]. It is therefore possible that results obtained with cell suspensions will not be valid for attached cells.

This report describes a method of making fluorescence measurements on attached cells with an ordinary spectrofluorimeter. The cells used, mouse neuroblastoma C 1300 clone 41A3, will grow both in suspension and attached to a surface. Fluorescence measurements using the probes ANS*, DC and NPN were carried out on suspended and attached cells. The results indicate that attached cells have a membrane organization which is different from that of cells in suspension.

2. Materials and methods

2.1. *Chemicals*

8-Anilino-1-naphtalene sulfonic acid (Mg–salt) and phenyl–α-naphtylamine were purchased from Eastman and dansyl cadaverine from Sigma Chemical Company. All probes were used without further purification. Ham's F 10 medium and foetal bovine serum were obtained from Flow Laboratories, and newborn calf serum from Wallenberg Laboratories, Uppsala. Penicillin and streptomycin were purchased from Glaxo.

2.2. *Cell cultures*

Mouse neuroblastoma C 1300 clone 41A3 was grown in Ham's F 10 medium supplemented with 10% newborn calf serum and 5% foetal calf serum. The medium also contained 100 units of penicillin and 50 μg streptomycin/ml. The cultures were kept at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was changed twice a week, and subcultures were made with 0.15% trypsin in a phosphate-buffered salt solution.

2.3. *Cell preparations*

After 1–2 days in suspension culture, the cells were separated from the medium and washed three times with Hank's solution by centrifugation. The cells were suspended in Hank's solution to a concentration of 150 000 cells/ml. The viability exceeded 95% as determined by exclusion of Trypan Blue. This preparation was used either for experiments on suspended cells, or incubated for 20 min at 37°C in a Petri dish with 25 × 10 × 1 mm quartz plates on the bottom. During this period, the cells attached to the quartz plates, which were used for experiments on attached cells. After a wash, the cells were incubated for an additional period of at least 10 min in Hank's solution. All experiments were performed within 1.5 h after separation from the culture medium.

Abbreviations: ANS, 8-anilino-1-naphtalene sulfonic acid; DC, dansyl cadaverine; NPN, phenyl–α-naphtylamine

2.4. *Fluorescence measurements*

All experiments were made with a Farrand Mk-1 spectrofluorimeter equipped with a ORTEC-Brookdeal 9511 quantum photometer and 5032 detector head. For measurements on attached cells, a special cuvette holder was used (fig.1). The quartz plate with the cells was inserted against one of the side-walls in the cuvette with the cells facing the solution. After passage through a narrow slit, the excitation beam reached the cuvette which was tilted 10°. Experiments on cell suspensions were made with a standard cuvette holder. The recorded fluorescence intensity was in the same order of magnitude with both methods. The excitation and emission monochromator band pass was set at 10 nm, except for measurements on cell suspensions, when the excitation monochromator band pass was 5 nm. The cuvette housing was kept at 37°C by means of circulating air.

For polarization measurements, Polaroid HNBP filters were used. The polarization of fluorescence, P, was calculated according to the formula

$$P = \frac{I_{vv} - I_{vh}\dfrac{(I_{hv})}{I_{hh}}}{I_{vv} + I_{vh}\dfrac{(I_{hv})}{I_{hh}}}$$

where the first index denotes the plane of polarization of the exciting light filter (vertical or horizontal) and the second index that of the emission filter.

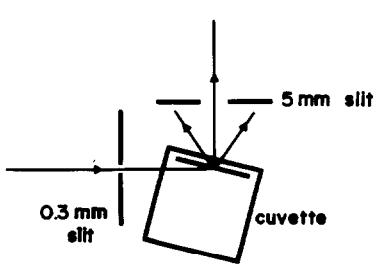

Fig.1. The cuvette position during measurement on attached cells. The cells are grown on a quartz plate inserted into the cuvette, which is tilted 10°. The extra slits are inserted to minimize scattering due to refraction of the exciting beam in the cuvette walls.

Attached cells were labelled with NPN by incubation with 1 μM NPN in Hank's solution for 30 min in a Petri dish under occasional agitation. In all other cases, 10 μl of each probe (from stock solutions) was added directly to the cuvette containing 2 ml Hank's solution. Stock solutions were prepared as follows: ANS was dissolved in Hank's solution to a concentration of 0.2 mM or 2 mM. NPN (4 mM) in ethanol was mixed 1:20 with Hank's solution. One part of 0.1 M DC in acetone was mixed with 100 parts of Hank's solution. This turbid mixture was allowed to separate overnight, and the clear supernatant containing 0.18 mM DC was used.

ANS was excited at 380 nm and the fluorescence was measured at 470 nm. The wavelengths used for NPN and DC were 340/410 nm and 335/505 nm, respectively. Results were expressed as relative fluorescence, calculated as the ratio between the fluorescence from bound-probe and the light-scattered by the cells before the probe was added. The same wavelengths were used for both measurements. All measurements were corrected for the fluorescence of free probe.

3. Results and discussion

3.1. *Measurements with NPN*

In order to make comparisons between measurements on suspended and attached cells, it is necessary to estimate the number of cells in the light path in each case. As a reference, the light scattered from the cells was measured before addition of the probe. To test if this signal was a valid measure of the cell number, cells were incubated in 1 μM NPN. This neutral, hydrophobic probe gives a very strong fluorescence with 41A3 cells, and the intensity is the same in a suspension of intact cells as in a cell homogenate. It is therefore assumed that NPN thoroughly penetrates the cell, binding to its hydrophobic constituents. As can be seen in table 1, the relative NPN fluorescence was about the same in the two preparations. Equivalent results are obtained if the scattered light is measured at the wavelengths used for ANS and DC. It is reasonable to assume that the relative fluorescence is a valid measure of the fluorescence of bound-probe/cell.

Table 1
Polarization of fluorescence and relative fluorescence for 41A3 cells

Probe		Suspended cells	Attached cells	Significance
NPN 1 μM	Rel. fluorescence	70.3 ± 5.0 (5)	62.6 ± 7.0 (6)	n.s.
	Polarization	0.083 ± 0.001 (5)	0.076 ± 0.002 (6)	$0.05 > p > 0.01$
ANS 10 μM	Polarization	0.219 ± 0.006 (6)	0.107 ± 0.028 (5)	$0.01 > p > 0.001$

Values given are the mean ± SE. The number of experiments is recorded within parenthesis. Significance was tested with Student's *t*-test.

3.2. *Measurements with ANS and DC*

The anionic probe ANS is known to bind to proteins and to membrane lipids. It has been used to label the membranes of living cell and, rather than penetrating the cell membrane, it is considered to be located in the lipid–water interface [1,3]. The probe DC is believed to bind to anionic sites in biomembranes [6].

Since it reflects the rate of rotation of the probe molecule, the polarization of fluorescence is used to measure the microviscosity of the environment of a probe [2,7]. The fluorescence polarization of the penetrating probe NPN was almost the same for suspended and attached cells, while the membrane-specific probe ANS showed a large difference in the polarization of fluorescence (table 1). It seems that ANS is more firmly bound to the sites of suspended cells than to the binding sites of attached ones. However, without fluorescence lifetime measurements, the results concerning fluorescence polarization must be considered as preliminary.

In fig.2 it can be seen that the initial incorporation of both probes was largest in the suspended cells, and that it reached equilibrium faster in suspended than in attached cells. This can be interpreted as a more 'open' membrane structure in suspended cells, that is, a conformation with easily accessible binding sites. These sites could, at least in the case of ANS, consist of a more fluid structure of the membrane lipids, since ANS has been shown to reflect the melting of a lipid structure by an increased fluorescence [3].

Besides structural changes in the cell membrane, changes in surface potential can affect the binding of charged probes [4]. As seen in fig.2, the attached

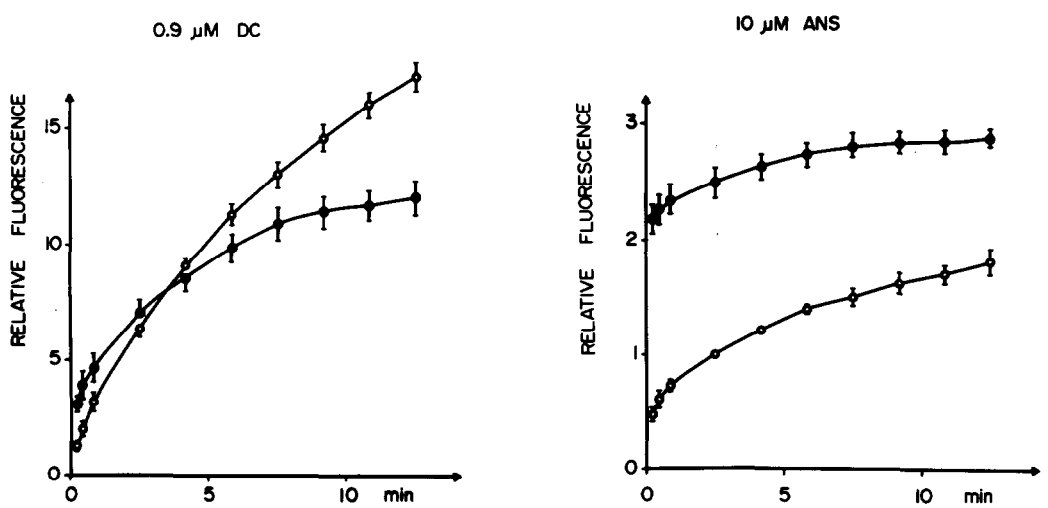

Fig.2. The relative fluorescence as a function of time after the addition of probe. The bars indicate the SE of three experiments. (o–o) Attached cells. (•——•) suspended cells.

cells bound a smaller proportion of ANS and a greater proportion of DC than suspended cells. It is possible that this reflects a more negatively charged cell surface.

These results clearly indicate that the attachment of a cell to a surface changes the properties of its cell membrane, although it is not possible to determine whether the observed effects on the binding of fluorescent probes reflect structural or electrical alterations of the cell membrane or both.

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