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Light-induced permeabilization and Merocyanine 540 staining of mouse spleen cells

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Merocyanine 540 (M540) is a potential-sensitive, hydrophobic dye that preferentially incorporates into the 'fluid' domains of cellular membranes, distinguishing between hemopoietic cells according to their differentiation state. A bright staining with M540 is usually achieved by UV illumination of the cells during staining. We show by flow cytometric analysis that: (1) staining is greatly enhanced by UV illumination of mouse spleen cells before addition of the dye; (2) UV treatment causes an increased permeability toward propidium iodide and intracellular fluorescein as well; (3) the increment in M540 fluorescence precedes permeabilization to propidium iodide, while the latter precedes leakage of fluorescein. We also describe an overshoot and accelerated recovery of M540 fluorescence after photobleaching by a 514 nm laser beam. It is suggested that penetration of M540 to the more fluid inner membrane structures explains the fluorescence increment in both experiments.

Introduction

Merocyanine 540 (M540) is an anionic, lipophylic, fluorescent dye primarily applied in membrane potential studies [1]. Both the absorption and fluorescence spectral properties of M540 are sensitive to changes in membrane potential, by influencing the partition of the dye between the medium and the membrane, its distribution between the positive and negative side and the equilibrium between highly fluorescent monomeric and quenched, aggregated molecules [1,2].

M540 incorporation is 'biased' toward fluid-like [3] and/or cholesterol-free phospholipid [4] domains of the membrane. Permeability of cells to M540 varies with regard to differentiation state [5–7]. Staining is markedly enhanced upon illumination of the cell suspension by UV light during staining [7] and eventually leads to cell death, through a mechanism not completely understood [5–7]. The latter phenomenon was used for selective in vitro killing of leukemic cells and referred to as 'M540-mediated photosensitization' [8]. Marine hemopoietic cells could also be distinguished on the basis of their different susceptibility to M540-mediated photosensitization [5].

In this communication we report flow cytometric and fluorescence recovery after photobleaching (FRAP) data relevant to this phenomenon. Our main finding is that UV illumination induces permeabilization of cells also in the absence of the dye, with a consequent enhancement of staining with various fluorescent markers, including M540.

Materials and Methods

Normal and Rauscher virus-infected Balb/c mouse spleen cells were used in the experiments. Preparation of cell suspension, inoculation of virus was performed as described elsewhere [9]. Merocyanine 540 (M540, Serva) stock solution 91 mg/ml) was prepared in ethanol/water (1 : 1, v/v), and stored in the refrigerator, protected from light. Staining of cells was achieved by adding 2.5 μl of dye solution to 100 μl of cell suspension ($1 \cdot 10^7$/ml in phosphate-buffered saline, PBS, pH 7.4). This sample was diluted with PBS to 1 ml and directly analysed in the flow cytometer (without washing), or measured in the FRAP apparatus (with or without washing of the cells). For FRAP analyses 5 μl of the $1 \cdot 10^7$/ml cell suspension was dropped on a slide, covered with a coverslip, sealed with paraffin and measured within 30 min.

Abbreviations: M540, Merocyanine 540; FDA, fluorescein diacetate; PI, propidium iodide.

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Propidium iodide (PI, Sigma Chem. Co.) was used at 20–50 μg/ml concentration, from a 1 mg/ml stock solution (in water).

Fluorescein diacetate (FDA, Koch-Light Ltd.) was dissolved in acetone to make a $1 \cdot 10^{-4}$ M stock solution. 10 μl of this solution was injected to 1 ml of the cell suspension ($1 \cdot 10^6$/ml) and the sample analysed continuously for 15 min The nonfluorescent ester FDA readily enters live cells due to its hydrophobicity. Strongly fluorescent fluorescein becomes liberated inside the cell by nonspecific esterase. A quick leakage of fluorescein (i.e., no or low intracellular fluorescence signal) is an indication of increased membrane permeability. which is usually considered as a symptom, or preclude, to cell death (see Ref. 14).

UV treatment of cells. 1 ml cell suspension ($1 \cdot 10^7$/ml, unstained) was placed under a 15 watt Germicid lamp (max. output at 254 nm) at a distance of 10 cm, and illuminated for 10 min.

Flow cytometric measurements. The argon-ion laser of a Becton-Dickinson FACS III apparatus was tuned to 514 nm for M540, M540-FDA and PI-stained samples, and to 488 nm in the case of FDA-PI stained cells. Forward light scattering (proportional to size) and fluorescence intensity signals are stored in a multichannel amplitude analyser and represented as distribution histograms or in case of correlated analysis of two parameters, as scattergrams (dot-plots), where each dot represents a cell positioned in a coordinate system of the two parameters (e.g., light scattering vs. fluorescence, or two fluorescence signals of different colours). The laser output was set to 400 mW. M540, fluorescein and PI fluorescence were detected through a 520 nm cut-off filter, 540 nm interference filter (Melles Griot), and 620 nm cut-off filter, respectively. PI-stained samples were run at low speed (200 cells/s).

Lateral mobility measurements. The fluorescence recovery after photobleaching apparatus built in this laboratory is basicly the same as described in Ref. 20. Components of the system are an argon ion laser (ILA-120-1, G.D.R., tuned to 514 nm and operated at maximum nominal output of 700 mW); a pair of planparallel flats to obtain bleaching and attenuated, monitoring beams; Fluoval (Carl Zeiss, Jena, G.D.R.) fluorescent microscope; RCA 8850 (U.S.A.) photomultiplier; and ICA 70 (KFKI, Hungary) multiscaler. The recovery curves were analysed according to Ref. 11. The radius of the illuminated spot was approx. 1 μm.

Results

Prolonged viewing of M540-stained cells under the fluorescent microscope produced a conspicuous enhancement of membrane fluorescence without any obvious alteration in the pattern of staining, similarly to what was described in Ref. 7. This phenomenon is especially spectacular in the shorter wavelength range, as is shown in Fig. 1. PI was also included in the staining solution to distinguish cells permeabilized also to PI.

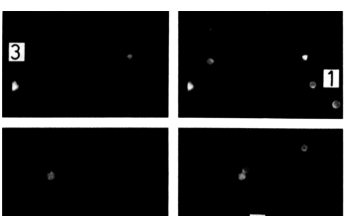


Fig. 1. Fluorescent photographs of mouse spleen cells incubated with 25 μg/ml M540 and 20 μg/ml PI, before (left pictures) and after 1 min illumination through the dichroic mirror adequate for UV excitation (right pictures, taken of the same field as their left pair). Numbers (1–3) indicate cells becoming M540-positive (1), M540 + PI-positive (2) and cells which were PI-positive before irradiation already (3).

Fig. 2 shows that UV treatment of cells prior to addition of the dye significantly increased stainability of normal spleen cells with M540, relative to untreated control cells. UV illumination of the dye solution before staining did not promote staining of cells (data not shown). Rauscher virus-infected erythroblasts were stained more readily than normal cells (in agreement with Ref. 6) in the absence of UV illumination and their UV treatment did not further enhance staining.

Fig. 3 shows that UV-treated normal and Rauscher cells become heterogeneously permeable also to PI, a dye generally regarded as a fluorescent alternative to the Trypan blue viability test [12,13]. Cells without UV treatment were > 95% PI-negative (not shown).

UV-treated cells became leaky to fluorescein in the fluorescein diacetate (FDA) hydrolysis test [14] also in a heterogeneous manner (Fig. 4). In a correlated (two-color) analyses of FDA/M540 double-stained UV-treated normal spleen cells three subpopulations were resolved: FDA − /M540 + (along the y axis); less or more FDA + /M540 − (along the x axis) and a heterogeneous population of FGDA + /M540 + cells. The latter cells were practically absent before UV treatment. Thus, subpopulation of the FDA-positive (fluorescein-inpermeable) cells still present after UV treatment have already become permeabilized to M540.

When UV-pretreated cells were stained with FDA and PI, the PI-positive cells still accumulated fluorescein at a normal rate (Fig. 5), in contrast with the results of FDA/M540 staining (Fig. 4). This suggests

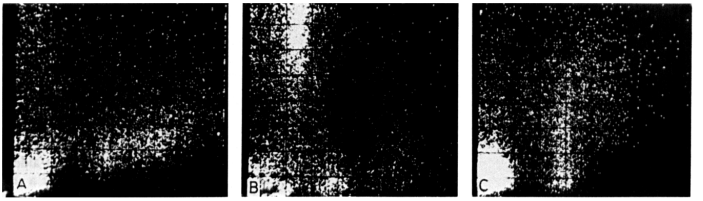
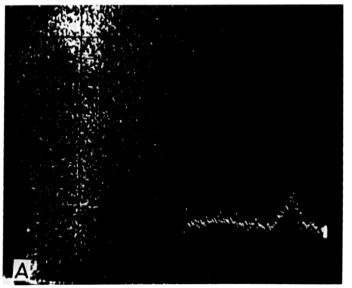
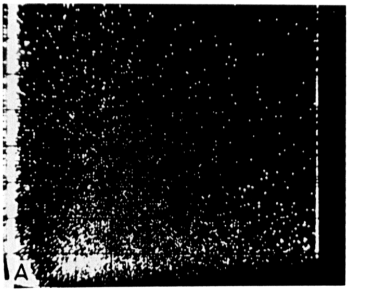


Fig. 2. Scattergrams of forward light scattering (abscissa, x) vs. fluorescence (ordinate, y) analysis of normal (B, C) and Rauscher virus-infected (A) mouse spleen cells, stained with 25 μg/ml M540. Before staining, cells were either illuminated with UV light (B) or not (A, C). For virus-infected cells scattergrams were identical with or without (A) treatment. Relative fluorescence amplification gains were 4 (A), 1 (B) and 16 (C).





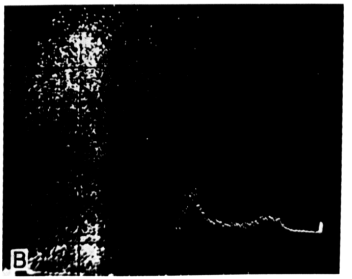


Fig. 3. Scattergrams of light scattering (x) vs. fluorescence (y) analysis of normal (B) and Rauscher virus-infected (A) spleen cells, stained with 20 μg/ml PI after UV treatment. Data collection was performed 5 min after addition of the dye. The insets are fluorescence intensity distribution histograms, corresponding to the scattergrams.

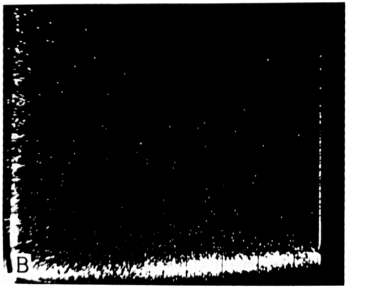


Fig. 4. Scattergrams of two-color FDA (x) vs. M540 (y) fluorescence analysis of normal spleen cells before (B) and after (A) UV illumination. Analysis was started 10 min after the addition of the dyes, applied at 10^{-6} M (FDA) and 25 μg/ml (M540) concentrations.

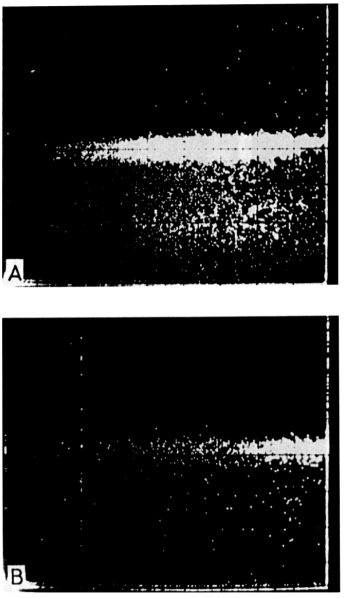


Fig. 5. Scattergrams of two-color FDA (x) vs. PI (y) fluorescence analysis of UV-treated normal spleen cells, 5 (A) and 10 (B) min after addition of the dyes, applied at 10^{-6} M (FDA) and 25 μg/ml (PI) concentrations.

that leakiness of most of the UV-treated FDA/M540-stained cells to fluorescein (Fig. 4) could not be solely the result of illumination, but also a consequence of M540 staining.

Acquisition of PI-positivity was a gradual process. Fig. 6 demonstrates that a subpopulation of cells exhibited moderate PI fluorescence in the beginning, then gradually merged into the population with maximal PI content (the latter population still accumulated fluorescein at a normal rate, see Fig. 5). According to Fig. 1, permeabilization to M540 precedes the collapse of permeability barriers to PI, i.e. intensive M540 staining occurs before any PI uptake.

Performing fluorescence after photobleaching (FRAP) experiments [11,15] on cells stained with M540 we observed further phenomena which were apparently in close connection with those described above. 'Recovery' to a much higher level of fluorescence than the original ensued after a short bleach (Fig. 7). The overshoot (1) was observed only in the case of samples stained with high concentrations of M540 (above 10 μg/ml) and, (2) using maximum laser output; (3) could be reproduced by repeated bleaching (Fig. 7C); (4) was frequently accompanied by a slow return of fluorescence intensity to a plateau exceeding the prebleach-level ('rebound' phenomenon, Fig. 7B) and (5) was not seen on cooled sample or after fixation with glutar aldehyde (data not shown). Certain cells showed rather a steady increase in fluorescence (Fig. 7D) after bleaching instead of pronounced overshoot. Diffusion constants in the order of 10^{-8} cm^2/s were calculated for the quick 'recovery' curves in contrast with much lower values (around 10^{-9} cm^2/s) calculated from 'normal' recovery curves in the absence of the overshoot effect (Fig. 7A).

Discussion

Cellular or experimental parameters determining the M540 dye uptake of cells are of practical importance,

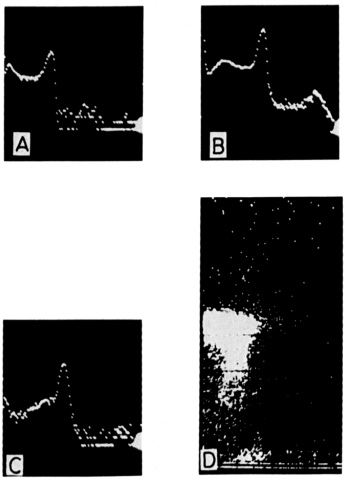


Fig. 6. Fluorescence intensity distribution histograms (A–C) of UV-treated normal spleen cells, 5, 10 and 15 min (A, B and C, respectively) after addition of PI at 50 μg/ml concentration. (D) Scattergram (x: light scattering; y: PI fluorescence) of C. The histograms are shown in semilog representation of the cell number.

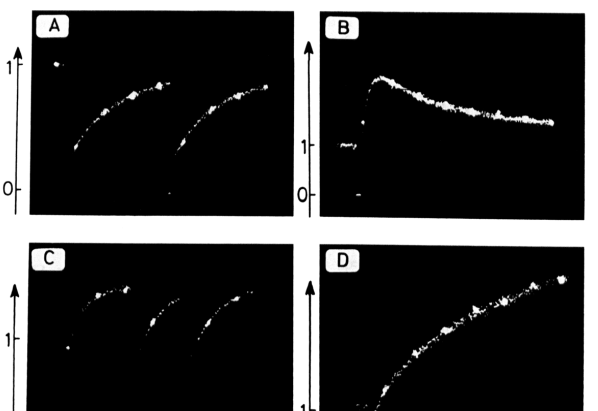


Fig. 7. Fluorescence recovery curves of M540-stained normal spleen cells. (A) Normal curve, obtained regularly at low dye concentrations (< 10 μg/ml) or when cells were washed after staining. (B–D) Different types of 'overshoot' effect in cells stained with 25 μg/ml M540 and analysed without washing of cells from excess dye. Bleachtime: 100 ms; the distance between two adjacent, amplified dots corresponds to 3.8 s.

since M540 distinguished between normal and leukemic, or immature hemopoietic cells [5,6]. Selective killing of leukemic cells by illumination during staining, was also reported and referred to as 'M540-mediated photosensitization' [8].

The data presented in this communication suggest that UV or intensive visible (laser) illumination of cells permeabilizes them to M540 and to some other fluorescent dyes as well and]mediation' of this effect by membrane-bound dye is not required. The dramatic difference in FDA hydrolysis between M540-stained, and UV-treated and PI-stained, UV-treated cells can be explained with the superposition of M540-toxicity on light-induced permeabilization. The leftward transposition of the scatter signals of highly M540-positive cells in Fig. 2 can indicate either diminishing refractive index of swelling cells [16] or may be due to increased axial light loss of M540-saturated cells, or both.

Regarding dye penetration the following sequences of postillumination events can be drawn: M540 → PI → fluorescein, i.e., cells get permeabilized to M540 first and to fluorescein last. Staining of cells with M540 is probably governed by f actors closely related to those determining the uptake of another dye, $N\epsilon$-dansyl-L-lysine [17]. Both of these processes were shown to be a (still reversible) prelude to cell death [4,17].

The 'dynamics' of heterogeneity with regard to PI staining can be explained supposing either (a) that membrane permeabilization proceeds in a gradual manner, or (b) the slow increase of fluorescence in the 'lower' PI-positive subpopulation may be due to the collapsing of chromatin structure from dye-excluding to a more dye-accessible conformation, as a consequence of membrane permeabilization and entrance of PI.

Staining of human erythrocyte ghosts with M540 is governed by phospholipid membrane asymmetry according to Refs. 19 and 20. Accessibility of lipids normally residing in the intracellular leaflet of the membrane, is prerequisit to staining. The enhanced staining of UV-treated cells and the overshoot effect in FRAP experiments could be the consequences of M540 entering into the cytoplasm and incorporation to the membrane from within. Alternatively, either or both of these phenomena may be related to the direct translocation of the dye to the inner membrane leaflet [18]. It is possible that the reported fluid-like domain-preference of M540 [3] is more readily satisfied by the less rigid phospholipid structure of the inner side [21], yielding an incre-

ment in the amount of fluorescing dye molecules in the membrane. Conceivably, the inevitable change in membrane potential following permeabilization also contributes to increased M540 binding, either directly or by its influence on transbilayer phospholipid mobility [22,23].

The slower recovery in the FRAP experiments (Fig. 7A) probably represents the lateral diffusion of M540 in the outer leaflet, while the quick recovery (Fig. 7B and C) can correspond to motion of the dye in the inner leaflet of the membrane (and possibly in other intracellular membrane structures as well).

The overshoot of fluorescence 'recovery' could be explained by light-induced permeabilization of the cellular membrane to M540 available extracellularly in abundance, supposing that both the high $h\nu$ energy of UV photons and the high number of (visible) photons supplied by the laser beam permeabilize the membrane perhaps by photochemical reactions or heat generation. We noticed, however, a significantly increased Hoechst 33342 uptake immediately after UV illumination (data not shown), instead of a decrease described for hyperthermia treatment [24], arguing against gross heat effects.

The 'rebound' phenomenon, i.e. the decrease of fluorescence intensity after recovery frequently seen after overshoot, can reflect the lateral diffusion of the dye from the illuminated spot to other parts of the inner leaflet. The practically unlimited number of repeated identical overshoot-rebound curves is also in agreement with this explanation.

There are methodological consequences of both our flow cytometric and FRAP results. As regards viability determination by (fluorescent) dye exclusion and FDA tests, the all-or-non rule widely held (including this laboratory, see Ref. 16) should be revised: the heterogeneity of PI staining implies that permeabilization is not an either-or feature of the cellular membrane (in accordance with Ref. 25), and different dyes are not interchangeable with respect to diagnosis of membrane barrier function. An inference of the FRAP data presented here is that the bleaching light may induce permeabilisation and transitory fluidization, which influence the apparent recovery rate of lipid probes, but may not be detectable in the case of the slower protein lateral mobility measurements [26].

The prevailing interpretation of the biological effects of UV irradiation is based on the DNA-damaging effects of UV light (see, for example, Ref. 27), although progressive swelling of UV-treated (red blood) cells was described earlier and explained by inactivation of ion pump mechanisms [28]. The above demonstration of membrane permeabilization upon UV treatment of nucleated cells implies that this effect is to be considered in case of findings like UV irradiation-induced stimulation of DNA-mediated transformation [29], induction by light, of oncogenic viruses [30] or malignants transformation of cells [31].

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