Intracellular phthalocyanine localization: confocal laser scanning microscopy studies

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ABSTRACT

Phthalocyanines (Pc) are promising second-generation photosensitizers for the photodynamic therapy (PDT) of cancer. We report on the tetrasulfonated aluminum phthalocyanine ($AlPcS_4$) localization in cultured Chinese hamster lung cells studied by means of confocal laser scanning microscopy (CLSM). In these cells AlPcS4 was found in granules surrounding Golgi apparatus and in the peripheral cytoplasmic region. Peripheral Pc-containing granules partially coincided with the acidic cellular compartments. The effect of irradiation with light on Pc intracellular distribution was also studied. In the Pc-free medium disruption of some Pc-containing granules was observed followed by appearance of Pc fluorescence in the cell plasma membrane, the nuclear envelope and the near-nuclear region. When cels were irradiated in the presence of Pc in external medium a drastic increase of membrane permeability to Pc was observed, followed by Pc binding the cell plasma membrane, nuclear envelope and some structures in the cytoplasm. Diffusive Pc fluorescence in the nucleus was also observed. The implication of observed Pc redistribution caused by irradiation with light for the PDT protocol is discussed.

1.INTRODUCTION

During last ten years a new modality of cancer treatment has been developed, called photodynamic therapy (PDT).[1-3] It is based on a phototoxic action of certain drugs that selectively accumulate in tumor tissues. The most widely used sensitizers are so called hematoporphyrin derivative and its most active fraction Photofrin II (see reviews [2,3]). Recently a second generation of photosensitizers was introduced which have a high absorption in the red region of optical spectrum (about 700 nm) where the light penetration depth in tissues is highest. Amongst the drugs of this generation the most intensely studied ones are phthalocyanines (Pc).[4-6] The best photodynamic efficiency is achieved for metallocomplexes of phthalocyanines with diamagnetic metals.[7-10] To obtain Pc with high water solubility, sulfonation procedures are usually used that introduce 1 to 4 sulfogroups into the molecule.[11] The efficiency of photodynamic cellular damage and intracellular accumulation depends strongly on the degree of sulfonation and even on the mutual position of the sulfogroups on the molecule.[8-10,12] The highest uptake in vitro was displayed by the lower sulfonated species, while this order was reported to be reversed in vivo.[12] Pc was observed in granules in cell cytoplasm, probably lysosomes[13], but they were not directly identified. No similarity of the intracellular fluorescence patterns was found for $AlPcS_2$ and Di-OC_6 (stains the mitochondria and endoplasmic reticulum), while they resembled each other for the $AlPcS_2$ and FITC-labeled low-density lipoproreins.[14]

Presently the mechanism by which the sulfonated Pc is internalized in cells is not entirely clear. Here we present data on intracellular localization of $AlPcS_4$ in cultured cells obtained by means of confocal laser scanning microscopy. In order to identify the cellular organelles containing Pc we used acridine orange (AO) for staining of acidic cellular compartments (lysosomes and endosomes) and C_5-DMB-ceramide for staining of the Golgi apparatus. The effect of exposure to light on the intracellular Pc fluorescence distribution was also studied.

2.MATERIALS AND METHODS

Phthalocyanine

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Commercially produced $AlPcS_4$ (a gift of Dr.H.Seidlitz) and $AlPcS_4$ produced in Moscow Institute of Organic Intermediates and Dyes were used. A stock solution of $AlPcS_4$ in PBS (10^{-2} M) was kept at 4°C in darkness. The loading solution for cells: 10^{-5} or 10^{-4} M Pc in MEM (minimum essential medium) with 3% of fetal calf serum (FCS).

Cell line

Monolayer growing Chinese hamster lung cells (CHL) were used on the third day of logarithmic growth.

Cell staining procedures

$AlPcS_4$. Cells were washed twice with MEM medium containing 3% FCS, incubated at 37°C, 0.05% CO_2 for 30 min. - 6 h, washed twice with Pc-free medium and either stained with additional fluorochromes or measured without delay.

Acridine orange. Stock AO solution (3 μg/ml in PBS) was diluted with MEM 1:2. Cells were washed twice with AO-free medium and placed into the loading AO solution for 30 min. This procedure resulted in red AO fluorescence in cellular acidic compartments. Then washed twice with the medium and measured.

C_5-DMB-ceramide. This fluorophore is a fluorescent BODIPY lipid that stains the Golgi apparatus of living cell. The staining procedure is described by Pagano et al.[18] Staining takes approximately one hour and results in green fluorescence of the cell plasma membrane, the nuclear envelope and some structures in the cytoplasm, and red fluorescence of the Golgi apparatus.

Hoechst. Stock Hoechst solution (1 mg/ml in ethanol) was diluted 1:200 with MEM. Cells were washed twice with MEM and placed into the loading Hoechst solution for 15 min. at room temperature. This procedure results in bright Hoechst fluorescence in cell nucleus.

Laser scanning confocal microscopy

Intracellular Pc localization was studied at the confocal laser scanning microscope (CLSM) made by Leica Lasertechnik, Germany and based on the inverted fluorescence microscope (Leica Fluovert FU) and two lasers: argon-krypton laser (488, 568 and 647 nm) and argon laser (351 nm). C_5-DMB-ceramide and AO fluorescence was excited by the 488 nm argon line of the cw Ar-Kr laser and registered simultaneously in green (528 nm< λ <542 nm) and red (λ >580 nm) channels, respectively. Hoechst fluorescence was excited by 350 nm argon line and registered at λ>410 nm. Pc fluorescence was excited by the 647 nm krypton line and registered at λ >665 nm. The power of the laser light at the sample was chosen at nondamaging level. The Leitz oil-immersion 63^x objective with N.A. 1.4 was used. Axial resolution of the confocal microscope was about 1 um.

3.RESULTS AND DISCUSSION

3.1. Pc intracellular localization during incubation in darkness

$AlPcS_4$ was found in cytoplasmic granules of diverse sizes (Figs.1,2). Frequently some of Pc-containing granules formed a "necklace" pattern tightly surrounding the cell nucleus. No Pc fluorescence was ever observed in the cell plasma membrane or the nucleus. Intracellular Pc distribution was qualitatively the same for incubation times from 30 min. up to 6 h and for both Pc concentrations of the loading solution. The only difference observed was the brightness of the granules that was observed to increase with incubation time as well as with external Pc concentration.

No colocalization of C_5-DMB-ceramide and Pc fluorescences was observed (Fig.3) indicating that Pc was not located in the Golgi apparatus. Pc-containing granules were found in the close vicinity to the Golgi complex, sometimes even interlaced with it. In principle we do not exclude the possibility that Pc is present inside the Golgi apparatus but its fluorescence is quenched due to the interaction with C5-DMB-

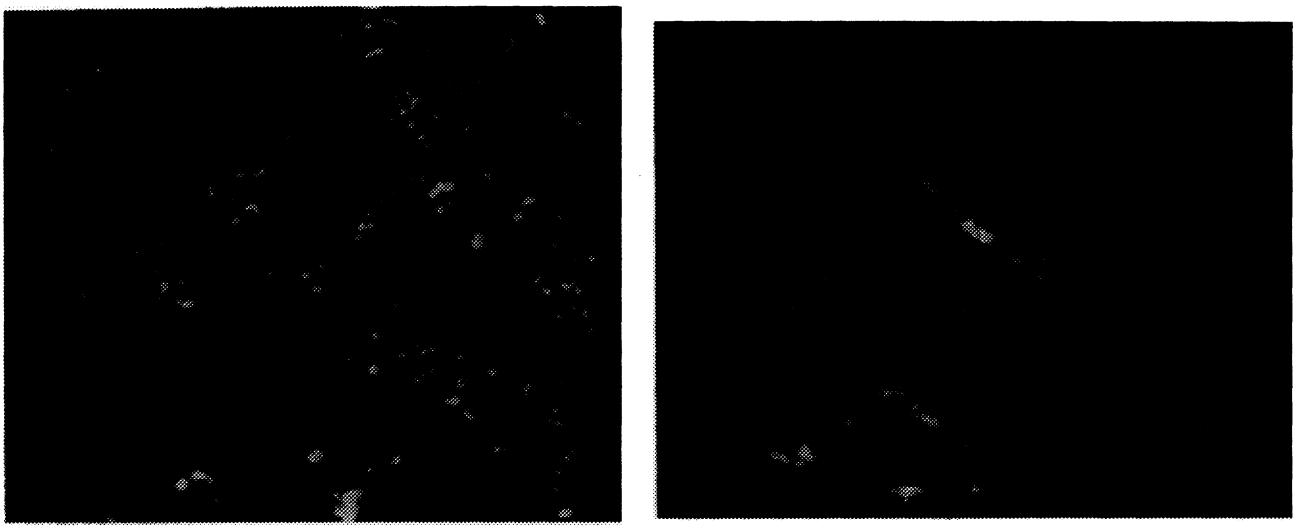

Fig.1a,b. Localization of tetrasulphonated aluminum phthalocyanine (Pc) fluorescence in CHL cells. a)Cells were incubated in 10^{-5} M Pc for 5 h at 37 °C; b)Cells were incubated in 10^{-4} M Pc for 3 h at 37 °C. The observation plane of the confocal microscope was chosen close to the suface of the cover glass with the monolayer. Note the Pc granules positioned at the end of a long thin strand of the fibroblast cytoplasm (center of Fig.1b).

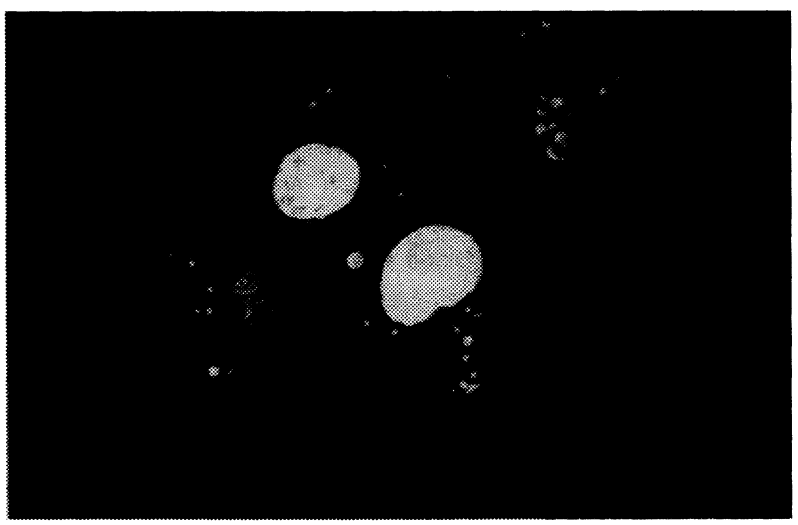

Fig.2. Localization of tetrasulphonated aluminum phthalocyanine fluorescence in CHL cells. The incubation procedure was the same as in Fig.1b. Cells were additionally stained with Hoechst to make nuclei visible.

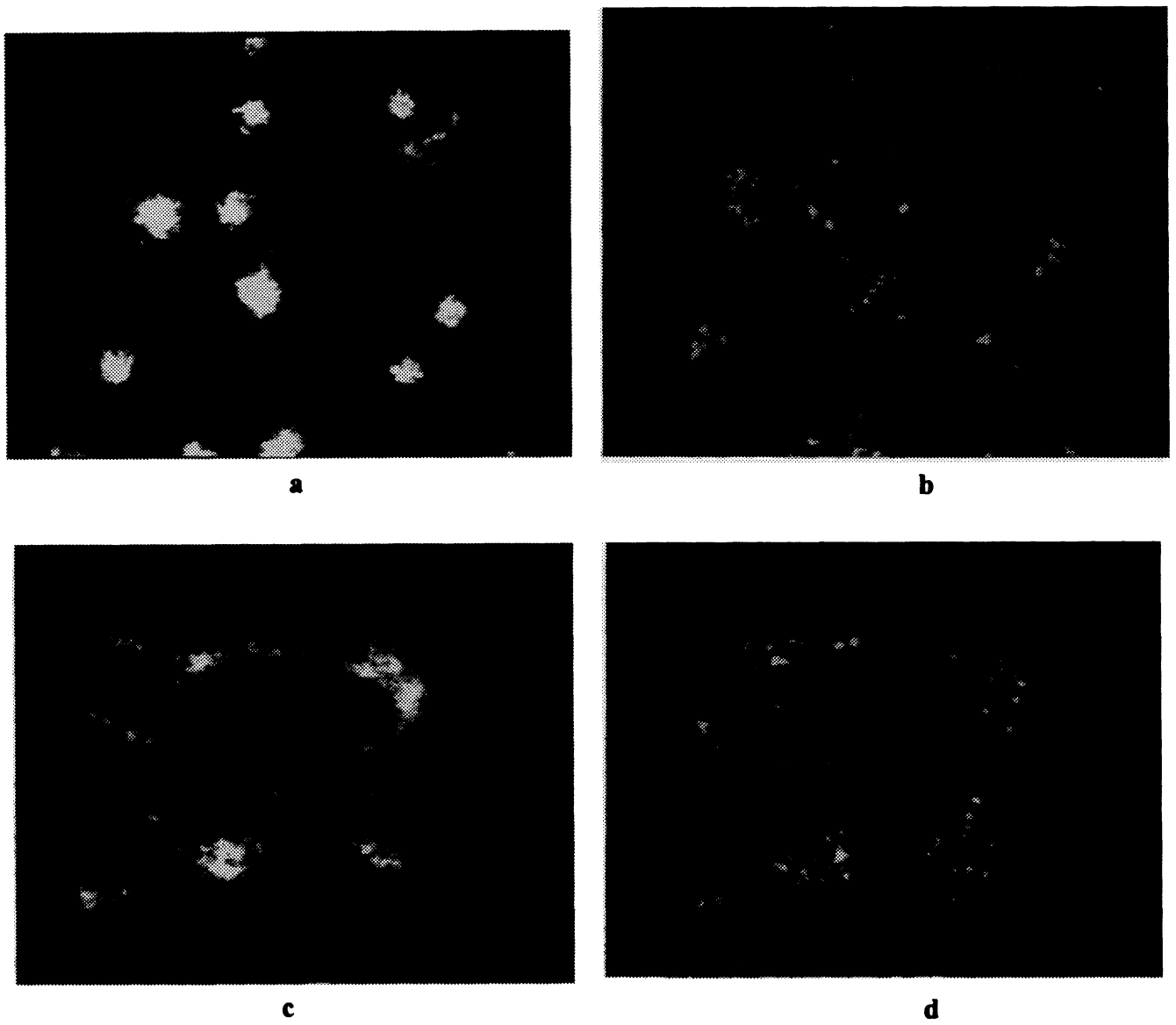

Fig.3. Patterns of C_5-DMB-ceramide (a,c) and Pc (b,d) fluorescences in CHL cells. Cells were incubated in 10^{-4} M Pc for 3 h and stained with C_5-DMB-ceramide. Images of C_5-DMB-ceramide and Pc fluorescences were measured at the same plane for each site.

ceramide. However the reproducibility of the data obtained for double-stained samples with different Pc concentrations and incubation times as well as the similarity of the patterns for the double-stained samples and samples stained only with Pc seem to indicate that this is not the case.

The data obtained for samples double-stained with AO and Pc appeared to be more complicated. Depending on the cell individuality, incubation time and concentrations of Pc and AO in the external medium three different patterns were observed: a bright granular Pc fluorescence pattern and no notable AO red fluorescence (data not shown); partial colocalization of Pc and AO red fluorescences (Fig.4) and, finally, no detectable Pc fluorescence and bright AO red fluorescence (see the lower left and right parts of Fig.4b,d). This variety of patterns indicated the mutual quenching of the Pc and AO fluorescences depending on the actual local concentrations of the fluorophores. It was also observed for the aqueous solutions of AO and Pc with different relative molar concentrations. This indicates that for the AO and Pc a high degree of colocalization is observed, but it is not possible to determine it quantitatively as long as the local concentrations of the fluorochromes are not known.

3.2. $AlPcS_4$ redistribution caused by irradiation with light.

The influence of light on the intracellular Pc distribution was also studied. To follow the light-induced damage the sample was continuously scanned by the 647 nm laser line and images of intracellular Pc fluorescence were taken every 30 s. The intensity of the laser beam was adjusted to cause the notable damage (as indicated by the Pc redistribution) during 10 - 15 min. of continuous scanning.

For the Pc-loaded cells in the Pc-free medium irradiation with light initially caused the increase of Pc fluorescence intensity without Pc redistribution. After 1.5-2 minuties of irradiation the disruption of some of the Pc-containing granules was observed and Pc fluorescence was appearing in the nuclear envelope, some near-nuclear structures and, weaker, in the plasma membrane (Fig.5). The appearance of the weak diffuse Pc fluorescence in the nucleus itself was also seen.

If irradiation was performed in the Pc-containing external medium, independent on the stage of the Pc intracellular accumulation, irradiation caused a rapidly increasing diffuse Pc fluorescence in the cytoplasm. This was followed by the appearance of Pc fluorescence in the nuclear envelope, in some cytoplasmic structures near the nucleus and in the cell plasma membrane (Fig.6). The diffuse intranuclear fluorescence was sometimes accompanied by the distinct spots. Eventually after about 10 min. the Pc fluorescence in the membrane structures of the damaged cell was even brighter than the Pc fluorescence in the external medium. In general the final Pc pattern was quite similar to the final stage observed for irradiated Pc-loaded cells in Pc-free medium.

It should be noted that the irradiation dose necessary to cause the Pc redistributuin was much lower in case of Pc presence in the external medium than in Pc-free medium.

Except for the diffuse fluorescence inside the nucleus the final intracellular Pc distribution after irradiation with light is quite similar to the one observed for the disulphonated Pc species (see elsewhere in this volume and also in [13]) and, in general, for the more hydrophobic sensitizers (Hp, HpD, Photofrin II, etc.) Considering the severity of the photodynamic damage it seems that this sensitizer localization is preferable as long as in this case more vitally important cellular structures get damaged. Thus we suggest that the observed $AlPcS_4$ redistribution caused by irradiation with light can be used for the enhancement of PDT efficiency of tetrasulphonated phthalocyanines that are reported to be present in the intracellular space inside the tumor tissue at least during the first hours after sensitizer injection.

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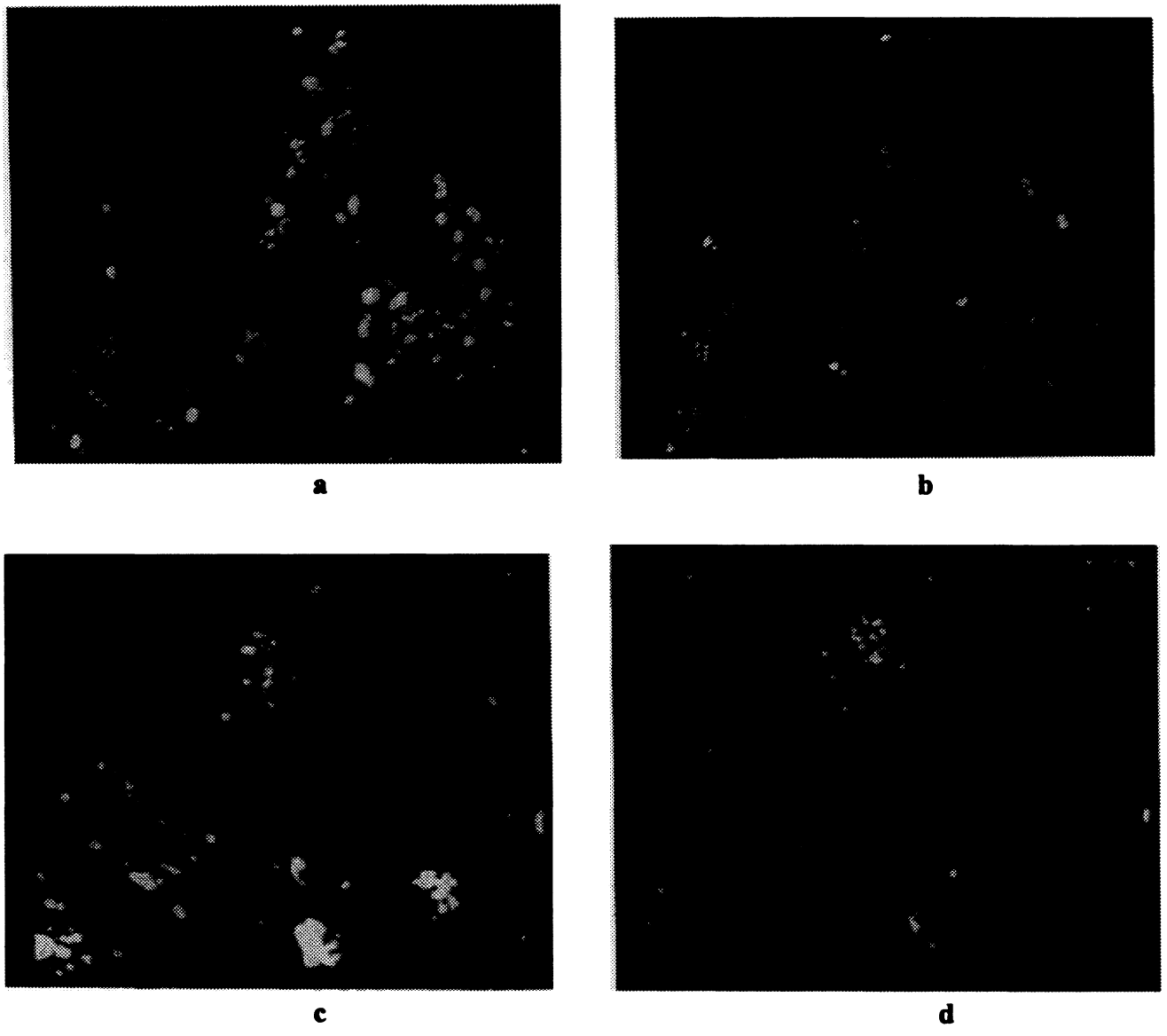

Fig.4. Patterns of AO (a,c) and Pc (b,d) fluorescences in CHL cells. a)Cells were incubated in 10^{-4} M for 3 h and stained with AO for 30 min.; b) Cells were incubated in 10^{-5} M Pc for 2.5 h and stained with AO for 30 min. Images of AO and PC fluorescences were measured in the same plane for each site.

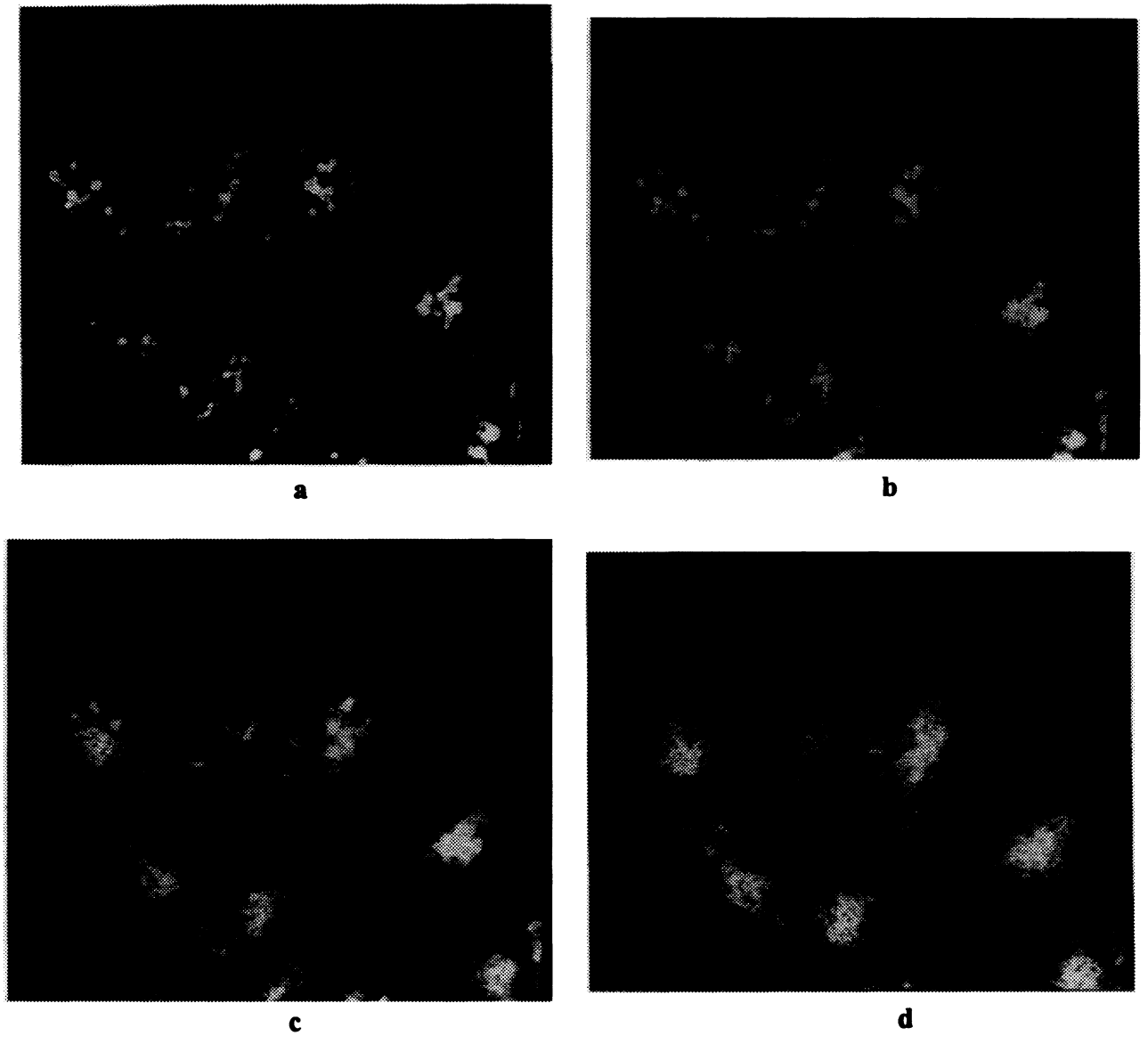

Fig.5. Appearance of Pc intracellular fluorescence caused by continuous irradiation with light (647 nm). Pc was present in the external medium (10^{-4} M in MEM medium with 3% FCS).

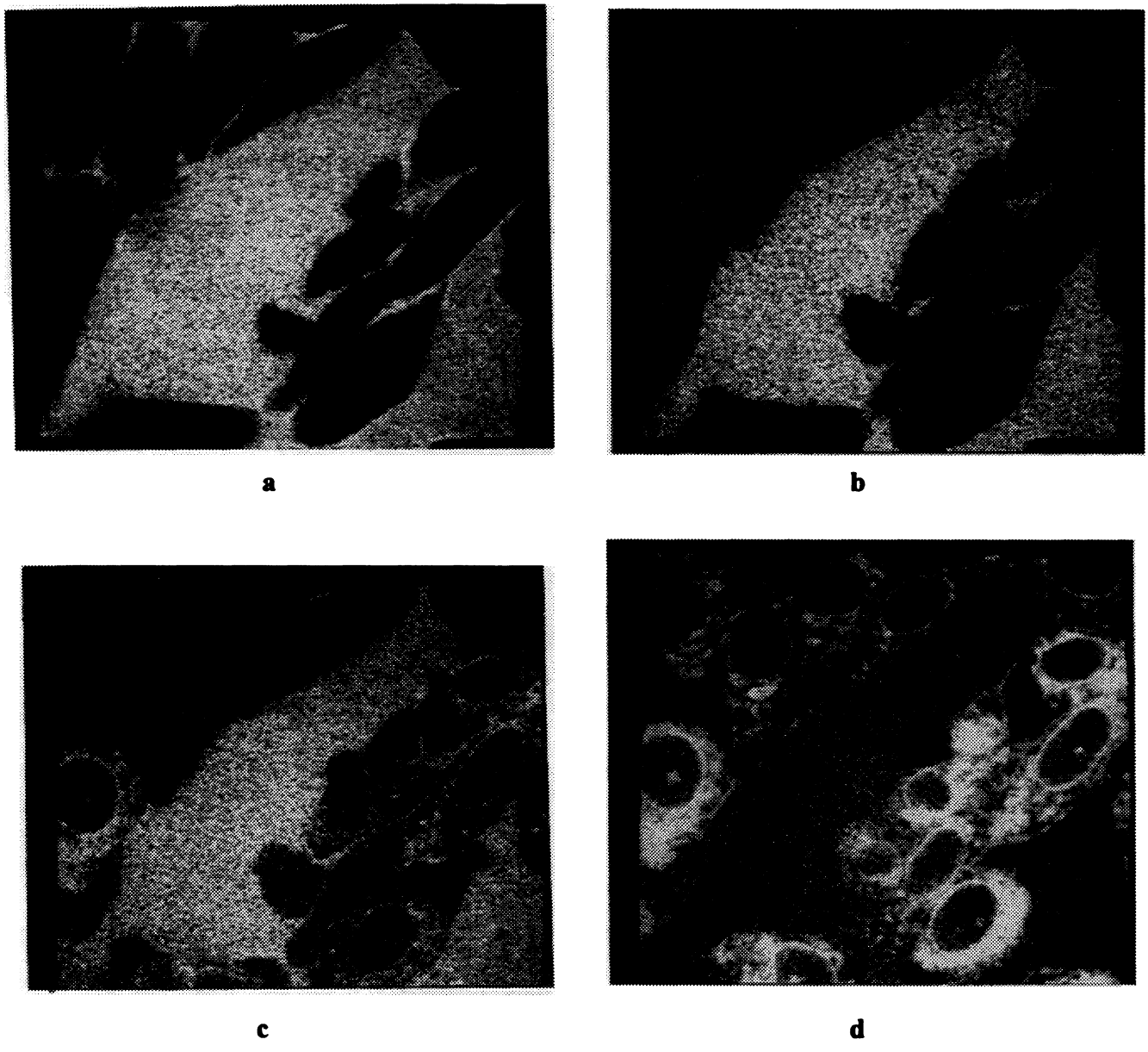

Fig.6. Redistribution of Pc intracellular fluorescence caused by continuous irradiation with light (647 nm). No Pc in the external medium. Cells were incubated in 10^{-4} M Pc for 6.5 h at 37 °C.

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