Spectral properties of phthalocyanines incorporated into resting and stimulated human peripheral blood cells

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Human peripheral blood cells stimulated by phytohemagglutinin (which serve as a model of cancerous cells) and resting cells were incubated in dimethyl sulfoxide solutions of various phthalocyanines. In order to diminish the influence of atmospheric oxygen the cells were embedded in a polymer (polyvinyl alcohol) film. Fluorescence spectra of the samples were measured over two regions of excitation wavelengths: at 405 nm (predominant absorption of the cell material) and in the regions of strong absorption of phthalocyanines (at about 605 nm and 337 nm). The intrinsic emission of cell material became changed as a result both of cells’ stimulation and of incubation of cells in dye solution. In most cases the stimulated cells when stained by dye exhibited higher long wavelength fluorescence intensity than resting cells. This suggests higher efficiency of dye incorporation into cancerous cells than into healthy cells. The absorption spectra of samples were also measured. The spectra of various phthalocyanines in incubation solvent, in polymer and in the cells embedded in polymer, were compared. The comparison of properties of the cells stimulated for different time periods enabled to establish the conditions of stimulation creating a population of cells incorporating a large number of sensitizing molecules.

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Abbreviations: Me2SO, dimethyl sulfoxide; PBMC, peripheral blood mononuclear cells; PDT, photodynamic therapy; Pcs, phthalocyanines; PHA, photohemagglutinin; PVA, polyvinyl alcohol.
Phthalocyanines (Pcs) are promising candidates for photodynamic therapy (PDT) because they have strong absorption in the Q band, located in a red region in which tissue is rather transparent (Rosenthal, 1991; Day et al., 1998; Osler et al., 2000; Frąckowiak et al., 2001). To be an efficient photosensitizer for PDT the dye should be selectively incorporated into the tissue. This means that it has to be much more efficiently introduced into stimulated cells (as a model of malignant cells) than into the resting cells. To select the type of Pcs which are most convenient for medical applications we compare, in this study, the absorption and fluorescence spectra of several Pcs in resting and stimulated peripheral blood mononuclear cells (PBMC).

In our previous investigations on PDT sensitizers (Frąckowiak et al., 1996; 1998; Ion et al., 1998; Frąckowiak et al., 2001a; 2001b; 2001d) we have used several spectroscopic methods. The investigated Pcs exhibited various, but always rather high, efficiency of triplet state generation. This property is important because, in most of the photodynamic reactions, the dyes participate in the triplet state (Rosenthal, 1991; Frąckowiak et al., 1996; 2001a). The absorption spectra of the dye incorporated into the cells provide low accuracy information because of small absorption amplitudes and perturbation of the spectra by light scattering. Therefore fluorescence methods are widely used (Pogue et al., 2001; Frąckowiak et al., 2001b; Dysart et al., 2002). The fluorescence of photosensitizers observed under a confocal microscope enables to establish the localization of dye in the cells (Cubeddu et al., 2000; Trivedi et al., 2000). Changes in the fluorescence of cell material due to illumination of samples were used in monitoring of cellular damage (Wood et al., 1997; Pogue et al., 2001; Dysart et al., 2002). The fluorescence spectra and yields of dyes depend on the sensitizer localization in the cells, but for a group of similar dye molecules, the cells stained to a higher degree usually show higher fluorescence intensity. Therefore, on the basis of fluorescence spectra it is possible to compare the degree of incorporation of various Pcs into the investigated cells.

The measurements of fluorescence spectra excited at two wavelengths: one efficiently absorbed by cell material and the second by the incorporated dye provide information about the modification which had occurred in cell material and about the dye incorporation. The change in time of cell stimulation makes possible to find appropriate conditions for obtaining stimulated cells incorporating a large number of sensitizing molecules.

To select the type of Pcs which are most convenient for medical applications we compared, in this study, the absorption and fluorescence spectra of several phthalocyanines in resting and stimulated peripheral blood mononuclear cells.

**MATERIAL AND METHODS**

The structures of the investigated Pcs are shown in Fig. 1. Solar Pcs was prepared by a two stage procedure starting with H2Pc(SO3-H)4 and diethynolamine as described by Ion (1999). The Pcs (substituted by Zn, and Al) were purchased from Sigma-Aldrich and used without further purification. Stimulation and staining of PBMC were carried out as described previously (Frąckowiak et al., 1996; 2001c).

Whole heparinized human blood samples remaining after routine analysis were used for the experiments. The purity and number of mononuclear cells (monocytes and lymphocytes) were established using flow cytometry. One part of the sample was stimulated using PHA (phytohemagglutinin HA 17, Wellcome, England) at a concentration of 10 µg/ml over a period of 1 h or, in some experiments, for 3 h or 6 h always at 37°C. The second part of the sample was not subjected to stimulation. A half of the stimulated and half of resting cells were stained, unstained halves serving as control samples. Dye solution at a concentration of 1 ×
10^{-4} \text{ M} \text{ in } 99.5\% \text{ dimethyl sulfoxide (Me}_2\text{SO) + 0.5\% H}_2\text{O (denoted in the text as Me}_2\text{SO} \text{) was added to a suspension of mononuclear blood cells (lymphocytes with low admixture of monocytes) containing } 3.6 \times 10^8 \text{ cells. The sample was incubated with dye for 1 h after adding 1 ml portions of dye solution to a such volume that 1 ml of the final suspension contained } 10^6 \text{ cells. The same volume of the solvent was added to the control sample. The stained cells were washed after the incubation, and then resuspended in unpigmented solvent.}

The cells were embedded in polymer polyvinyl alcohol (PVA) film as described previously (Frąckowiak et al., 2001). The absorption spectra of such samples were measured with respect to PVA film using a Shimadzu UV-V-1601 UV-VIS spectrophotometer. The fluorescence spectra of the same dyes embedded in PVA were measured in arbitrary units, the same for every set of experiments, at the same intensities of exciting light, width of slits etc., by means of an Hitachi F4500 instrument.

The PBMC samples embedded in PVA were photochemically stable enough to maintain their spectra unaltered for the duration of experiments.

**RESULTS AND DISCUSSION**

Absorption spectra of all the investigated phthalocyanine dye molecules in PVA films (Fig. 2) exhibited a strong band in the 600–760 nm region (Q band) and a weaker (B
band) over in the 300–400 nm range. A similar absorption was observed in the incubation solvent (Me₂SO) (not shown). This is a typical absorption pattern of Pcs making them good candidates for PDT (Frąckowiak et al., 2001b; 2001d). The main fluorescence emission maxima, at 337 nm excitation, were located in the 650–800 nm region (Fig. 3).

From a comparison of the fluorescence properties of Pcs in Me₂SO (not shown) and in PVA (Fig. 3 and Frąckowiak et al., 2002) it follows that the change in surroundings of the dyes on incorporation of their molecules into a rigid matrix causes some perturbation of the fluorescence yields and spectra. The ratio of the fluorescence yields in Me₂SO and in aqueous PVA solution was, e.g., about 5.9. The result obtained show that the efficiency of incorporation of a dye into cells can be evaluated only by comparing their fluorescence band intensities in incubation solvent and in the cells. It is useful to take into account also the absorption and photoacoustic spectra.

The absorption spectra of the resting and the activated PBMC, stained by Solar Pc and unstained, embedded in PVA (Fig. 4) show that it is not easy to establish the efficiency of dye incorporation on the basis of absorption spectra alone since the differences in absorption values for samples subjected to various treatments are low and measurements are perturbed by light scattering. In some cases, e.g., ZnPc, the dyes which, as judged on the basis of their emission or photothermal spectra (Frąckowiak et al., 1996; 1998; 2001b; Ion et al., 1998), were selectively incorporated into stimulated stained cells and reference resting cells, exhibited only slight differences in long wavelength absorption. From our previous investigations (Frąckowiak et al., 2001a) it is...
known that ZnPc exhibits a very high population of triplet state. From this point of view it is a promising candidate for sensitizer in PDT.

Both, the addition of PHA and dye have some influence on the absorption spectra of the cell material but the effects are not easy to interpret without monitoring the cell structure under microscope (Cubeddu et al., 2000; Trivedi et al., 2000).

The fluorescence spectra of PBMC stained by Solar Pc excited at 610 nm (in the region of strong absorption of the dye) (Fig. 5a) and excited in the region of cell material absorption (Fig. 5b) were taken for the cells stimulated by PHA over various periods of time. The fluorescence of stimulated cells first increased, when

Figure 5. Fluorescence spectra in PVA film of cells stained by Solar Pc.

a, excitation at 605 nm, measured in the region of dye emission; b, excitation at 405 nm, measured in the region of cells emission. Curve 1, resting cells; curves 2 and 3, cells activated for 3 h and 4–6 h, respectively. Incubation conditions in Solar Pc solutions were identical for all samples.

the stimulation time was extended from 1 h to 3 h, but started to became decreased after 6 h of stimulation (Fig. 5a). In the first hour of stimulation more dye was embedded into resting cells than into stimulated cells as shown by fluorescence of the resting cells which was lower than that of the stimulated cells (not shown). The difference in fluorescence intensity between stimulated and resting samples was the largest as a result of activation for 3 h (Fig. 5a).

Emission in the 500 nm region (excited at 405 nm where absorption of cell material is stronger than that of the dye) (Fig. 5b) was due predominantly to intrinsic fluorescence of PBMC. At least part of cell intrinsic fluorescence in the 450 nm region is due to, nicotinamide adenine dinucleotide (NADH) emission (Pogue et al., 2001) reduced as a result of illumination. This emission decreases in the presence of oxygen (Pogue et al., 2001), therefore it can be intensive in the case of our cells embedded in PVA. Some flavin emission can not be excluded, either. The excitation energy transfer from excited stained cell macromolecules to B band of Pcs is possible. In this case the emission of incorporated dye should increase.

The short wavelength fluorescence of our samples (Fig. 5b) was the highest for both, stained and unstained cells, after 3 h of activation but it was very similar to the emission of unstained resting cells.

The incubation of resting cells with dye caused a decrease in fluorescence intensity. Solar Pc emission in this region is very weak (Fig. 3), therefore this decrease should be related to a change in properties of the cell material. It is known that at various excitation and observation of emission wavelengths the ratio of malignant to healthy cell emission can be different (Yang et al., 1997; Brancalone et al., 2001). On excitation at about 300 nm the tumor tissue fluorescence is stronger, whereas at longer wavelengths it is weaker (Yang et al., 1997). A shift of emission maxima was also observed (Yang et al., 1997; Brancalone et al.,
The differences in emission between malignant and healthy tissue are probably due to alterations in protein structure. The possibility that some special proteins could be formed in malignant tissue should not be excluded. In our experiments, the differences between resting and stimulated model cells might have been caused by perturbation of protein structure not only by activator, but also by dye molecules and the incubation solvent (Me₂SO). When the intrinsic fluorescence of cells is strongly influenced by the dye incorporation, one can suspect that the dye is toxic and that, even after it is expelled from resting cells they could still be in disturbed state. The influence of Solar Pc on intrinsic PBMC emission is very complex. The emission from resting unstained cells was much higher than from stained cells. The activation by PHA caused an increase in emission from stained cells, but this increase was dependent on activation time. It seems that some of the procedures applied caused an increase, while the effect of others was to reduce emission. The superposition of all the effects gave a complex dependence of emission on the presence of activator. On the basis of emission taken at one wavelength of excitation it is not possible to evaluate the contributions from various intrinsic chromophores to the observed emission. Most of the previous measurements of tissue fluorescence have been carried out at shorter wavelengths (Brancaleon et al., 2001) than those used by us for excitation. It is known (Lakowicz, 1999) that some originally nonfluorescent substances can become strongly fluorescent when they are bound to proteins. A change in protein conformation can have a strong influence on the shape and intensity of the emission spectrum. We can therefore regard the observed change in short

![Graph](image1)

**Figure 6.** Fluorescence spectra in PVA film of cells stained by AlPcCl II.

a, excitation at 680 nm; b, excitation at 405 nm. Curve 1, resting cells; curves 2, 3, 4, cells activated for 1 h, 3 h and 6 h, respectively.

![Graph](image2)

**Figure 7.** Intensity of main fluorescence maxima of activated cells, versus time of activation.

Curves 1 and 2, cells stained by AlPcCl II. 1, excitation at 680 nm, observation at 770 nm and 2, excitation at 405 nm, observation at 498 nm. Curve 3 unstained cells, excitation at 405 nm, observation at 498 nm.
wavelength fluorescence as evidence that the conformation of macromolecules become perturbed.

From fluorescence of AlPcCl II (Fig. 6a and Fig. 7) it follows that the longer time of activation had rather little influence on incorporation of this dye into activated cells. The difference between long wavelength band intensity of resting and stimulated cells was observed as soon as after 1 h of activation. Similarly as for Solar Pc, the time of incubation of cells had to be properly established to obtain higher efficiency of incorporation of dye molecules into malignant than into resting cells. Activation for 6 h gave the best result, but even after such a long incubation time, differences in the incorporation of dye into resting and activated cells were not very large.

Over the short wavelength region, the addition of AlPcCl II to resting cells caused a strong decrease of fluorescence (Figs. 6b and 7) as compared with unstained cells, showing that the changes were not due to dye emission. Both for unstained and stained activated cells the emission intensity was dependent on the activation time. It seems that, for AlPcCl II, not only PHA action but also dye addition have a strong influence on cell properties.

The influence of PHA on cell structure is evident from Fig. 8a presenting fluorescence of PBMC stained by ZnPc. In this case the dye fluorescence maximum (at 703 nm) was much higher for activated cells than for resting cells. This shows that the selectivity of dye incorporation into cells was very high. The maximum at 494 nm strongly increased as a result of cell activation, and its further increase is due to ZnPc addition.

For cells incubated with AlPcCl I (Fig. 8b) the difference in the emission of dye from activated and resting cells was low. The fluorescence maxima were mutually shifted suggesting differences in dye environments in these two types of cells. The maximum at 497 nm was the highest for resting unstained cells, and it decreased to a similar degree as a result of dye or/and activator addition.

CONCLUSIONS

It has been shown that the fluorescence of dyes incorporated into peripheral blood mononuclear cells can provide information about the efficiency of dye incorporation. One has to take into account the fact that the yield of fluorescence in incubation solvent and in cells may be different from that in the cells, but, for a set of dyes, it is possible to compare their selectivity and efficiency of incorporation.

The influence of PHA activator on intrinsic fluorescence emission of the cells, observed in
this study, could be expected because the activator should change the structure cell material in order to produce the model of a malignant cell. Optimal time of activation of the cells varied from one Pcs to another. This may suggest that for different types of perturbed malignant cells different Pcs could be suitable as sensitizers.

It is known (Yang et al., 1997; Brancaleon et al., 2001) that the intrinsic fluorescence of healthy cells differes from that of malignant cells, therefore this emission can be used as a marker of cell perturbation. Out of the Pcs investigated for possible PDT application, ZnPc seems to be the most promising because it seems not to be toxic for resting cells, exhibits very efficient incorporation into malignant cells and low level of incorporation into resting cells.
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