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EFFECTS OF PHOTOSENSITIZATION AND LOW-POWER HELIUM-NEON LASER IRRADIATION ON LIPOSOMES AND CELL MEMBRANES

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Abstract

Low-power He-Ne laser irradiation causes a well-defined and energy dependent cell destruction of *in vitro* cultured cell lines sensitized by hematoporphyrin derivative (HPD). The mechanism of this photosensitization was studied by measuring with polarization microscopic, scanning electron microscopic, and electron-spin-resonance (ESR) spectroscopic parameters. The cell damage caused by photosensitization and laser irradiation seems to be a complex process, however the biological membranes seem to be one of the primary targets. The energy of laser light causes rotation and resonance changes of macromolecules and the water molecules, resulting in an increased structural order of the submembraneous components in the living cells, detectable microscopically. During the photosensitization process, the red (630 nm) He-Ne laser light, during a one-photon energy activation, causes excitation of hematoporphyrin molecules to their triplet state. The excitation of HPD molecules results in a multi-step, free-radical generating effect, measured by ESR spectroscopy and studied by the ultrastructural changes of membrane organization and cell shape. Similar effects could be observed on *in vitro* lipid-water liposome membranes.

Key Words: Photosensitization, hematoporphyrin, He-Ne laser, liposome, polarization microscopy, topo-optical reactions, scanning electron microscopy, cell membrane, electron-spin-resonance spectroscopy (ESR).

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Introduction

Energy dependent He-Ne laser effects were studied previously on *in vitro* cultured cells and a well characterized dose-response curve (activation by lower energy levels, and cell damage caused by higher energies) has been mapped out (3, 4). Similar effects could not be observed by irradiation with a monochromatic but non-polarized and non-coherent red light source at the same energy output and wavelength (3). Using different photosensitizer dye molecules, the sensitivity of cells to the laser light could be increased experimentally (4). The same energy level (15 J/cm^2) caused stimulation of some *in vivo* cell functions (e.g., phagocytic activity, secretion of immunoglobulins etc.). The same dose of irradiated energy induced cell destruction after treatment of cells with different photosensitizers (4).

During the past several years, an increasing number of investigations has been published, focusing on the use of different photosensitizer dyes (26, 33) both for tumor therapy (16) and tumor localization (13, 22). The most suitable photosensitizers have proved to be the hematoporphyrins (HP) and their derivatives (HPD), of which the latter ones consist of complex mixture of porphyrins (22). Comparing the normal cells to the malignantly transformed forms, an increased accumulation of HP dyes inside the neoplasms can be observed (33). Irradiation of sensitized cells by low-power, laser light (e.g., He-Ne laser, 632.8 nm) causes a selective photodynamic effect, resulting in complex damage to the tumor cells (1, 4).

The discrepancy of the absorption spectra of HPD and the wavelength of activating laser light seems to be a physical paradox (9). Red laser light activation of HPD occurs at 630 nm, which is the lowest energy maximum absorption peak of HPD, causes a one-photon absorption of the molecule (maximal absorption range at 405 nm: Soret band) (6). If the HPD molecule is already in an excited state, two types of different reactions (Type I and Type II) have been reported. During these reactions free radicals are generated by an energy transfer from the excited triplet state of the HPD dye (15).

In the Type I reaction, the most important compounds are: the free radical form of the sensitizer, the

superoxide anion (O^{2-}) and the hydroxyl radical (OH^-) (2, 11). During the Type II reaction, the singlet oxygen ($^1O_2$) is the most important compound (2, 19, 21).

The cell membrane seems to be one of the primary targets of photosensitization (1, 8, 12). All of these free radical compounds mentioned above, can damage cell membranes (10, 23). In our experiments, we wanted to identify the primary target of the activating laser light, and determine the mechanisms of action on the membrane structure. Effects of the generated free radicals on membranes were studied during these experiments by polarization microscopy and scanning electron microscopy (SEM). Our ESR spectroscopic measurements suggest a multi-step process of HPD photosensitization with He-Ne laser irradiation. The generated free radicals seem to be secondary products of laser activated HPD-water complexes, as described in our previous study (27).

Materials and methods

Chemicals

Hematoporphyrin dihydrochloride (HP) (Cat. No. 5518, Sigma Chem. Co., USA) and hematoporphyrin derivative (HPD) prepared according to the method of Lipson *et al.* (22) were used as sensitizer. A stock solution (1 mg/ml) was prepared, stored frozen, and diluted just before use. The purity of HP and HPD were confirmed by thin layer chromatography (TLC).

Egg lecithin (phosphatidylcholine type XI-E, Cat. No. 2772, Sigma Chem. Co., USA) was also checked using TLC, and prepared for ESR spectroscopic and polarization microscopic measurements by the method of Kinsky *et al.* (20): 5.0 mg egg lecithin, with or without HPD at various concentrations (5-20 μg/ml) was dissolved in 0.5 ml chloroform and evaporated in a round flask under a N_2 gas stream in the dark.

The spin trap N-t-butyl-alpha-phenyl-nitrone (PBN) (Cat. No. 7263, Sigma Chem. Co. USA), 150 mM dissolved in 70% ethanol-water mixture was used, and treated with different concentration of HPD, dissolved in phosphate buffer saline (pH 7.4, 0.9% NaCl, 10 mM Na-phosphate) (PBS).

Light source

He-Ne laser (Type L.S.32, Quantum Physics, Division of Electro Nuclear Labs., USA) at the wavelength of 632.8 nm (red) and power output of 5.6 mW was applied. The irradiated area was a 3.0 mm diameter circle. Using continuous wave laser with constant power, the radiation energy is in direct proportion to the illumination time. The laser produces 1 Joule energy during 3 minutes, which is equal to 14.1 Joule/cm^2 energy output.

Cells and culturing

Mouse lymphoid cell line (Sp-2/o) (31) and fresh and preserved human red blood cells (HRBC) were used as *in vivo* targets.

The culture medium was RPMI-1640 (Flow-Labs. Ltd., England) containing 10% FCS (fetal calf serum, Hungarocalf, Mezöhegyes, Hungary) in a 5% CO_2 humidified atmosphere at 37°C (tissue culture incubator, Hereaus, Germany). Tissue culture plates (V-form, 96 well, Greiner, Nurtingen, Germany) were used for incubation and irradiation. The diameter of the wells containing 50.0 μl of culture media, corresponded to the diameter of the laser ray. The cells were incubated with 5.0-10.0 μg/ml HPD dissolved in RPMI-1640 minus FCS, for 3 hours, in the dark, then washed 3 times in PBS. 10^5 cells in 50.0 μl PBS were irradiated and three parallel samples were counted microscopically. The viability of cells was calculated by trypan blue dye exclusion (34).

Polarization microscopic measurements were performed on sensitized and irradiated red blood cells or lymphocytes, after toluidine blue topo-optical staining reaction of the cell membranes, according to the method of Romhányi *et al.* (30). After the laser treatment, cell smears were fixed in 0.5% isotonic glutaraldehyde-phosphate buffer (0.1M pH 7.4) for 30 minutes at room temperature, washed three times in PBS, and stained with 0.1% toluidine-blue (pH 7.4) for 5 minutes. To precipitate the toluidine blue molecules built into the membrane structure, the unrinsed smears were washed with 0.5% potassium ferricyanide-1.5% potassium iodide (w/v) mixture two times. The smears were coated with a thin film of gum arabic, containing 0,5% potassium ferricyanide-1.5% potassium iodide (w/v). The birefringence of the cells was measured in a polarizing microscope (OPTON Standard, Germany), between crossed polars. The membrane birefringence (optical retardation) changes were measured using lambda/4 compensator filter by the method of Köhler (29, 30), and counted on 1000-1000 cells from every sample.

Formal birefringence (without any staining) could be measured on artificial membranes, prepared according to the method of Romhányi *et al.* (29) by polarization microscopy. The pretreated egg lecithin was layered on glass plates in a 1-2 mm diameter area, one drop of PBS (pH 7.4) was put on the top of the lecithin layer and covered with glass plates. Artificial membranes (myelin figures) were "growing off" from the water-lipid boundary. Formal birefringence of native membranes was compared with samples, irradiated with laser light and/or photosensitized with HPD previously.

Scanning electron microscopic measurements were performed on human red blood cells and mouse lymphocytes (Sp-2/o myeloma cells), treated with HPD and

irradiated with laser light in 96 well tissue culture plates. The samples were fixed with 0.5% glutaraldehyde-phosphate buffer for 30 minutes, washed three times with PBS, and collected on a SELAS flotronics FM-25 filter membrane (0.8 μm pore size) by gravity filtration. Dehydration was carried out by passing a graded series of ethanol over cells, trapped on the membrane. The samples were critical point dried, and coated with gold-palladium before observation with a JEOL scanning electron microscope (JEOL 100C E.M. ASID-4, Japan).

ESR spectroscopic measurements were carried out using the PBN spin-trap, with different concentration of HPD in solutions and on HPD-PBN treated liposomes, prepared as described above. The lecithin film was vortexed with 1 ml PBS (pH 7.4) for 3 minutes at room temperature in the dark. The mixture was placed into a cuvette and irradiated in the spectrometer (Type ESR 220, Carl Zeiss, Jena, Germany) in the dark.

Results and Discussion

Polarization optical detection of membrane alterations

Changes of membrane birefringence by toluidine blue induced topo-optical reaction was observed on HPD treated and untreated mouse lymphoid cells (Sp-2/o) and human red blood cells (Table 1).

Laser irradiation of the untreated cells at 14-20 Joule/cm^2 energy output increased the anisotropy on both cell types. The viability of cells was over 95% at 0 and 1 hour after the laser exposure. He-Ne laser irradiation of the HPD pretreated Sp-2/o cells at the same energy level caused only a minimal decrease both of birefringence and cell viability, measured immediately after the treatment. However, the viability of the photosensitized Sp-2/o cells was reduced to 5 % at the end of a one hour period. The ultrastructural features of plasma membranes changed parallel with the cell viability: the cells lost the intensive radial birefringence, and the granular form "crystals", with decreased birefringence were detectable in the membrane structure. The HPD treated and laser irradiated red blood cells showed similar changes: the membrane birefringence decreased rapidly, measured by anisotropic toluidine blue staining.

Romhányi's topo-optical reactions are able to detect fine structural changes of molecular organization. The polar group of the toluidine blue molecules show an oriented binding to the acidic glycoproteins of cell surface, while their flat, apolar parts are intercalated between the lipid molecules. The rapid decrease of radial birefringence on the HPD treated cells suggests a molecular disorganization of plasma membrane. The appearance of "granular", optically oriented structures in the membrane of the irradiated lymphatic cells might indicate the presence of focal concentrations of altered lipid components.

Table 1: Anisotropy changes on *in vitro* cultured mouse myeloma (Sp-2/o) and human red blood cells (HRBC).

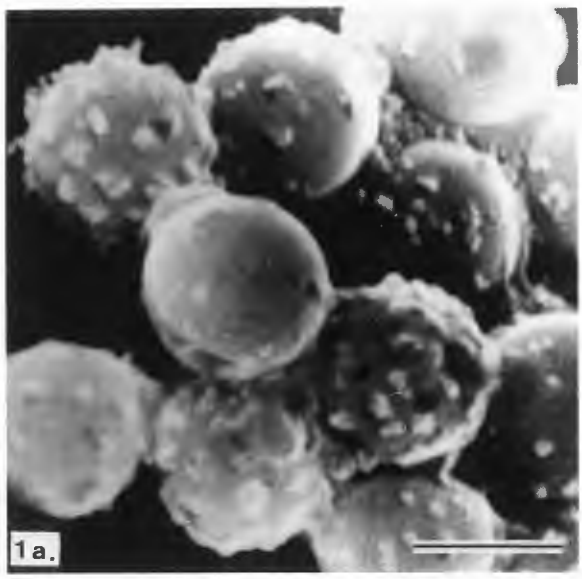
Treatment	Optical retardation (nm)			
	Sp-2/o		HRBC	
	0 hour	1 hour	0 hour	1 hour
Control	38 ± 3	38 ± 5	40 ± 3	40 ± 2
Laser	45 ± 5	39 ± 8	51 ± 2	40 ± 3
HPD + Laser	32 ± 9	5 ± 4	37 ± 6	0 ± 3

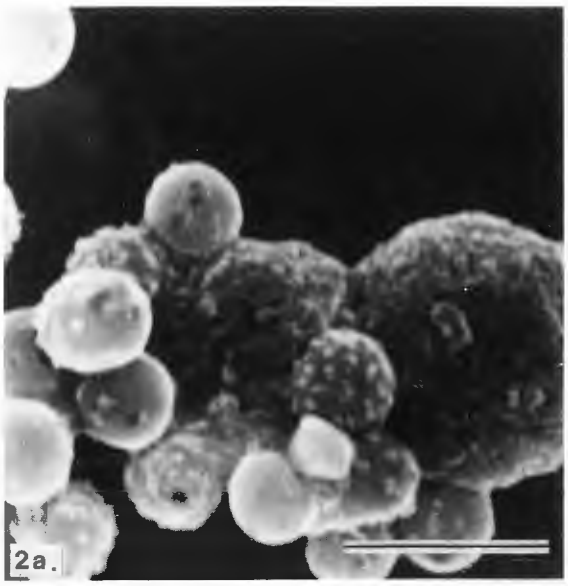
The anisotropy changes of 1000-1000 cells of 3 parallels of control, laser irradiated and HPD + laser treated samples were measured by toluidine blue (pH 7.4) potassium-ferricyanide topo-optical reaction, at 540 nm wavelength (data indicate x ± standard deviation). The birefringence of untreated control cells did not change during 1 hour period. Laser irradiation caused a reversible slow increase of birefringence. HPD pretreated cells after the laser irradiation lose the membrane birefringence during 1 hour period.

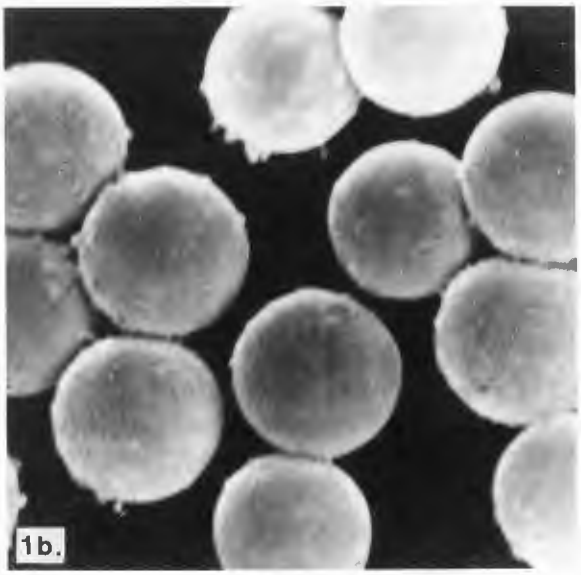
Different changes of the living cells caused by physical influences (e.g., radio- or photoirradiation) are well studied with various morphological examinations (24, 25). However, the details of these morphological findings can reflect only partially on the biological mechanism (28). Functional and morphological methods performed parallel on the same samples seems to be more advantageous to explain the possible mechanism of low-power laser effects, both on the living cells and *in vitro* models.

Scanning electron microscopic observations

SEM examinations were done on human red blood cells and *in vitro* cultured Sp-2/o mouse myeloma cells during our experiments. Old HRBCs (over 100 days) have an echinocyte-spherocyte conversion (caused by metabolic changes) (14), characterized by the rough surface on SEM pictures (5) (Fig. 1a). Immediately after the photosensitization (HPD and He-Ne laser irradiation by 14-20 J/cm^2), the surface of the red blood cells became "smooth" (Fig. 1b). These rapid morphological changes are related to the early alterations of membrane components (which are more visible on the old echinocytes, but could be observed also on the normal discocyte forms and cup-shaped somatocytes as well). The photodynamic effect of HPD and laser irradiation induces a free radical production which may cause saturation of the unsaturated fatty acid molecules and be responsible for the alterations of membrane proteins. These effects appear as the "rigidization" of membrane structure. Mouse myeloma cells, during the







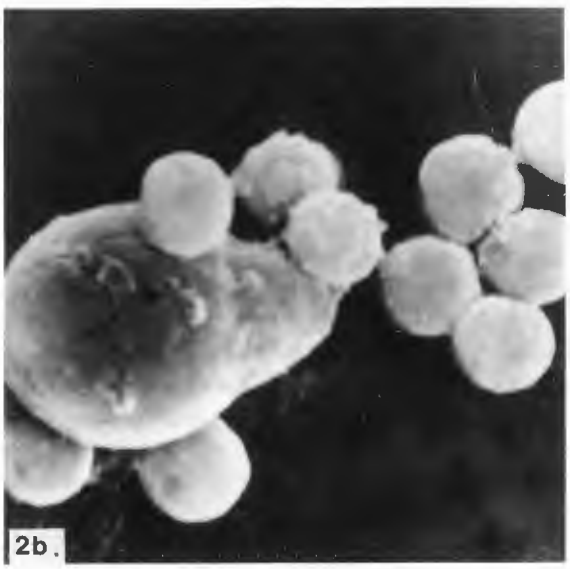


Fig. 1 (at left). Scanning electron micrographs of human red blood cells. **a**: untreated controls; **b**: photosensitized, He-Ne laser irradiated sample. The "old" HRBC-s showed a rough surface in case of untreated controls, while the laser treated cells became smooth. Bar = 5 μm.

Fig. 2 (at right). Scanning electron micrographs of Sp-2/o cells and HRBC-s. **a**: control; **b**: HPD treated, laser irradiated cells. Both cell populations show rough surface before, and smooth surface after the laser treatment. The round form of laser treated cells with smooth surface suggests an ultrastructural "rigidization" detected on sedimented samples. Bar = 10 μm.

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sedimentation process, show a flat form on SEM preparations (Fig. 2a). The HPD sensitized, laser irradiated Sp-2/o cells show a rounder appearance under SEM (Fig. 2b), similar to the findings on red blood cells. One hour after the laser irradiation, most of the photosensitized cells were lethally damaged, characterized by pycnotic appearance under SEM (results not shown).

We have demonstrated a well detectable plasma membrane rearrangement after the photosensitization process. The loss of fine structural organization of plasma membrane characterized by the topo-optical reaction and changes in the cell shape and plasma membrane surface investigated by SEM suggest molecular alterations in membrane molecules. This process could be

Table 2. Changes of native birefringence of artificial membranes.

Sample	Optical retardation (nm) 0 hour	1 hour
Native	60-100	60-100
Laser irrad.	50-80	60-100
HPD + laser	0-40	0-2

The lecithin-water artificial liposome membranes show an intensive formal birefringence. After single laser irradiation (14-20 J/cm^2), the molecular order became temporarily disorganized. The HPD photosensitization with laser irradiation causes total optical disorder of artificial membranes. The process developed continuously after the laser treatment in the samples. The 0 hour values were measured immediately after the laser irradiation.

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observed earlier than the dramatic decrease of cell viability after the He-Ne laser treatment. The question arises: is the delayed lethality after the initial cell membrane damage based on the functional "buffer capacity" of cytoskeletal structures (18), or is the laser irradiation only a starting effect of a "chain reaction" in the HPD sensitized membrane? To address this problem, experiments were designed using photosensitized artificial membranes.

Artificial model membrane experiments

No alterations in formal birefringence of liposome membranes were detected by polarization microscopy on the untreated control samples. The laser irradiated (but not HPD treated) liposomes ("myelin figures") show a partially increased birefringence on the water-lipid border with appearance of optically isotropic bands inside of the lipid membranes (Fig. 3b). These phenomena appeared temporary, and disappeared a few minutes after the single laser irradiation. HPD sensitized liposomes show a rapid and irreversible decrease of birefringence. (Table 2). The linear appearance of the native, (Fig. 3a) artificial lipid membrane changed to spherical and optically isotropic aggregations. This process also continued in time after the photosensitization and laser irradiation until the total structural decomposition of the artificial membrane structure was evident (Fig. 3c).

Stabilization of the "myelin figures" with cholesterol or proteins (e.g., BSA) did not alter the morphological changes observed on the liposomes (results not shown). To compare the morphological changes with the functional observations (ESR), the simplest composition of the liposomes seemed to be the most useful to characterize the targets of the initial steps of photosensitization. The results of our experiments performed on liposomes

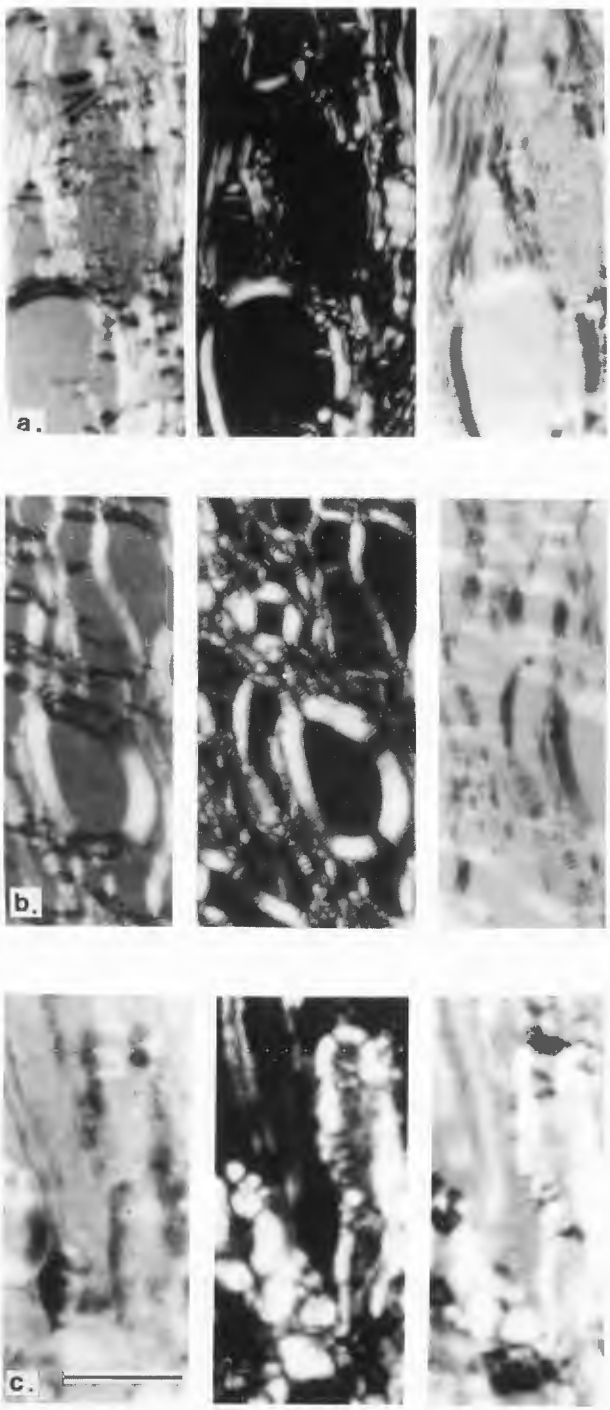

Fig. 3. Polarization microscopic appearance of lecithin-water artificial membranes prepared according to the method of Romhányi. **a**: untreated control; **b**: laser irradiation (15J/cm^2) causes a moderate increase of birefringence; **c**: HPD sensitized and laser irradiated sample loose the classic "myelin figure" appearance and decomposed optically. Bar = 100 μm.

presented similar phenomena observed in living cell membranes with similar appearance. The HPD sensitized and laser irradiated cells show similar time dependent membrane disorganization microscopically compared to the structural decomposition of artificial membranes. In both cases the laser activation of the HPD molecules, intercalated between the lipid molecules, starts a photo-induced "chain-reaction": the laser excited HPD molecules, even after the laser treatment, generate a multi-step free radical production (26, 27), which finally resulted in peroxidation of membrane lipid molecules.

Detection of the mechanism of photosensitization by ESR spectroscopy

ESR spectroscopic measurements were performed to clarify the physical process of free-radical generation from HPD during the photosensitization. The concentration of the excited HPD molecules and its products in a cellular system or in the liposomes are too low for direct ESR detection. The decreasing amount of lipid soluble, stable free radicals in HP or HPD sensitized, laser irradiated liposomes reflect the production of reactive radicals (27). No such effect has been observed in absence of either the sensitizer or the light.

An indirect ESR method (spin trapping) was used to clarify the compounds of free radicals, generated by HPD sensitization and laser irradiation. The differences in the ESR spectra of the spin-trap molecules (PBN) are dependent upon the trapped radical (17). In case of the HPD sensitized liposomes, the high background generated due to the large number of different trapped radicals makes it impossible to identify them. Irradiation of pure HPD-PBN solution caused well-defined spin adducts. On the basis of hyperfine splitting constants, three spin adducts (free radicals) have been detected. (Fig. 4).

By comparing our data (Fig. 4) to those in the literature (7, 23), we tried to identify these products. The 1 adduct arises first. Production of it depends on the HPD concentration; the illumination time and the water content of the solution (data not shown). Presumably this 1 adduct is the free radical form of sensitizer HPD molecule because the splitting constants exclude all of the oxygen radicals (17). Production of the 2 adduct depends on the concentration of the 1 adduct and is identified as a hydroxyl free radical. The 3 adduct appears after a few hours, but becomes dominant at this time. This is a stable nitroxide radical.

In our experiments, the He-Ne laser irradiation at 632.8 nm causes a one-photon induced activation of the photosensitizer HPD molecules in water solution. The free radical conversion depends on the degree of HPD molecules hydration. The excitation of HPD molecules is the first of the multi-step, time dependent free-radical

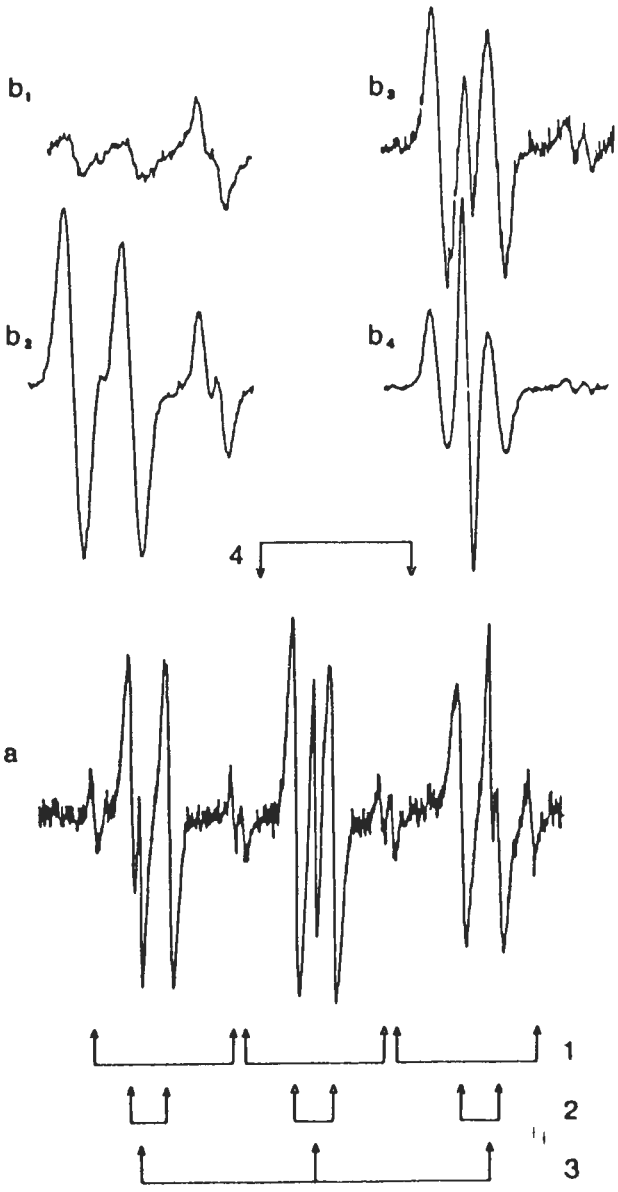

Fig. 4. The ESR spectra of the three (**a, 1-3**) spin adducts generated by laser excitation of HPD molecules, using PBN spin trap in 70% ethanol-water solution. **4** : this part of the spectrum is shown in the Figures b_{1-4}, which are the spectra of the adducts after 1 minute, (b_1); half hour (b_2); 2 hours (b_3); and half day (b_4), respectively. The excitation of HPD molecules (first adduct : 1) seems to be the initial reaction of this multi-step, time dependent free-radical conversion.

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conversion. The consequent formation of free radicals is responsible for the slow membrane damage which is earlier than the cell death, and appears as lipid disorganization in polarization microscopy and as increased mem-

brane rigidity by SEM investigations. The alterations of the cell membrane of the photosensitized cells seems to be one of the initial steps of cell destruction caused by HPD photosensitization.

References

1. Allison AC, Magnus IA, Young MR (1966) Role of lysosomes and cell membranes in photosensitization. Nature **209**, 874-878.

2. Athar M, Mukhtar H, Bickers DR (1988) Differential role of reactive oxygen intermediates in Photofrin-I-and Photophrin-II-mediated photoenhancement of Lipid peroxidation in epidermal microsomal membranes. J. Invest. Dermatol. **90**, 652-657.

3. Berki T, Németh P, Hegedüs J (1985) Effect of low-power, continuous-wave He-Ne laser irradiation on *in vitro* cultured lymphatic cell lines and macrophages. Studia Biophys. **105**, 141-148.

4. Berki T, Németh P, Hegedüs J (1988) Biological effects of low-power He-Ne laser irradiation. Lasers in Med. Sci. **3**, 35-39.

5. Bessis M (1973) Living blood cells and their ultrastructure. Springer Verlag, Berlin, 95-104.

6. Bodaness RS, Heller DF, Krasinsky J, King DS (1986) The two-photon laser induced fluorescence of the tumor-localizing photosensitizer hematoporphyrin derivative. J. Biol. Chem. **261**, 12098-12101.

7. Buettner GR (1985) Thyil free radical production with hematoporphyrin derivative, cysteine and light: a spin trapping study. FEBS Letters **177**, 295-299.

8. Cohen S, Margalit R (1985) Binding of hematoporphyrin derivative to membranes. Expression of porphyrin heterogeneity and effects of cholesterol studied in large unilamellar liposomes. Biochim. Biophys. Acta. **813**, 307-312.

9. Cowled PA, Grace JR, Forbes IJ (1984) Comparison of the efficiency of pulsed and continuous-wave red laser light in induction of phototoxicity by HPD. Photochem. Photobiol. **39**, 115-117.

10. Das M, Mukhtar H, Greenspan ER, Bickers DR (1985) Photoenhancement of lipid peroxidation associated with the generation of reactive oxygen species in hepatic microsomes of hematoporphyrin derivative-treated rats. Cancer Res. **45**, 6328-6330.

11. Davila J, Harriman A (1989) Photosensitized oxidation of biomaterials and related model compounds. Photochem. Photobiol. **50**, 29-35.

12. Delmelle M (1978) Retinal sensitized photodynamic damage to liposomes. Photochem. Photobiol. **28**, 357-360.

13. Dougherty TJ (1980) Hematoporphyrin derivative for detection and treatment of cancer. J. Surg. Oncology **15**, 209-210.

14. Elgsaeter A, Stokke BT, Mikkelsen A, Branton D (1986) The molecular basis of erythrocyte shape. Science **234**, 1217-1223.

15. Gollnick K, Franken T, Schade G, Dorhofer G (1970) Photosensitized oxygenation as a function of the triplet energy of sensitizer. Ann. NY. Acad. Sci. **171**, 89-107.

16. Gulliya KS, Pervaiz Sh (1989) Elimination of clonogenic tumor cells from HL-60, Daudi, and U-937 cell lines by laser photoradiation therapy: implication for autologous bone marrow purging. Blood **73**, 1059-1065.

17. Janzen EG (1980) A critical review of spin trapping in biological systems. In: Free radicals in biology. Vol.IV., Pryor WA (ed.), Academic Press, New York, 115-124.

18. Kellermayer M, Ludány A, Jobst K, Szücs G, Trombitás K, Hazlewood CF (1986) Cocompartmentation of proteins and K^+ within the living cell. Proc. Natl. Acad. Sci. USA **83**, 1011-1015.

19. Kimel S, Tromberg BJ, Roberts WG, Berns MW (1989) Singlet oxygen generation of porphyrins, chlorines, and phthalocyanines. Photochem. Photobiol. **50**, 175-183.

20. Kinsky SC, Haxby J, Kinsky CB, Demel RA (1968) Effect of cholesterol on the sensitivity of liposomes to the polyene antibiotic filipin. Biochim. Biophys. Acta **152**, 174-185.

21. Lion Y, Delmelle M, van de Vorst A (1976) New method of detecting singlet oxygen production. Nature **263**, 442-443.

22. Lipson L, Balders C, Olsen A (1961) The use of a derivative of hematoporphyrin in tumor detection. J. Natl. Cancer Inst. **26**, 1-8.

23. Moan J, Vistnes AI (1986) Porphyrin photosensitization of proteins in cell membranes as studied by spin-labelling and by quantification of DTNB-reactive SH-groups. Photochem. Photobiol. **44**, 15-19.

24. Mok YC, Pang KM, Au CY, Yew DT (1988) Preliminary observations on the effects *in vivo* and *in vitro* of low dose laser on the epithelia of the bladder, trachea, and tongue of mouse. Scanning Microsc. **2**, 493-502.

25. Nelson AC, Wyle HR (1985) Morphological changes in neutron irradiated red blood cells. Scanning Electron Microsc. **1985; IV**, 1623-1630.

26. Pooler JP (1989) Photooxidation of cell membranes using eosin derivatives that locate in lipid or protein to study the role of diffusible intermediates. Photochem. Photobiol. **50**, 55-68.

27. Pótó L, Berki T (1989) Investigation on the free radical producing effect of hematoporphyrin (A spin trapping study). Acta Physiol. Hung. **74**, 285-290.

28. Pourreau-Schneider N, Ahmed A, Soudry M, Jacquemier J, Kopp F, Franquin JC, Martin PM (1990)

Helium-Neon laser treatment transforms fibroblasts into myofibroblasts. Am. J. Path. **137**, 171-178.

29. Romhányi Gy, Molnár L (1974) Optical polarization indicates linear arrangement of rhodopsin oligosaccharide chain in rod disk membranes of frog retina. Nature **249**, 486-488.

30. Romhányi Gy, Molnár L, Németh A (1974) Ultrastructural differences in cell membranes of erythrocytes, myeloid and lymphoid cells shown by topo-optical reactions. Histochemistry **39**, 261-276.

31. Schulman M, Wilde CD, Kohler G (1978) A better cell line making hybridomas secreting specific antibodies. Nature **276**, 269-271.

32. Sheven C (1986) The shape changes of erythrocytes: new approaches to an old problem. Acta Histochem. (Suppl.) Jena **33**, 107-113.

33. Shulok JR, Klaunig JE, Selman SH, Schaffer PJ, Goldblatt PJ (1986) Cellular effects of hematoporphyrin derivative photodynamic therapy on normal and neoplastic rat bladder cells. Am. J. Path. **122**, 277-283.

34. Tennant JR (1964) Evaluation of trypan blue technique for determination of cell viability. Transplant. **2**, 685-687.

Discussion with Reviewers

D.T. Yew: What exactly did you mean by the term "rigidization? What subcellular mechanisms may be involved? How did this change in morphology relate to the common pathological event of swelling?

Authors: We explained in the paper the early effects of HPD-laser induced photoirradiation on the membrane structure examined both by morphological (SEM, polarization microscopic) and functional (ESR) methods. The observed alterations (the cells became smooth and round, the membrane anisotropy increased immediately after the irradiation) were also studied in an artificial membrane model, which gave information about the primary targets of photosensitization. The activation of the HPD molecules is followed by a multi-step, time-dependent free radical generation resulting in the oxidation (saturation) of the membrane lipid molecules.

This phenomenon could be described as "rigidization" of the membrane structure. These early alterations precede the drastic decrease of cell viability. The common pathological event of swelling is an energy defective state of cells, which is not in direct relation with the early membrane alterations observed, but could be a later step of cell death caused by photosensitization.

D.T. Yew: Why there has been such a drastic decrease in viability within such a short period of time?

Authors: The time-response curve of photosensitization on *in vitro* cultured mouse cell lines was already determined in our previous studies (Berki *et al.* Studia Biophysica (1985) **105**, 141-148). However, in the present paper our goal was to study the early alterations after photosensitization, before the cell death. The trypan blue dye exclusion test is an accepted method to determine cell viability, which gave us repeatable results in the 0 and 1 hour time interval after the photosensitization.

I.F. Miller: The liposomes used in this study are poor analogs for cells (were not stabilized with cholesterol, no proteins were present). Although it is interesting that the liposomes seem to behave in a manner similar to the cultured cells, this is no proof that similar mechanisms are operating.

Authors: The lecithin liposomes used to determine the mechanism of photosensitization are poor analogues of living cells, however, they gave correct information about the target of photosensitization because they were a homogenous complex of the photosensitizer, the lecithin and water. This simple model lets us observe parallel morphological (polarization microscopic) and functional (ESR) alterations. The liposomes observed were stable enough during a one hour period, and stabilization of them with cholesterol or proteins did not cause the morphological alterations but only made it impossible to identify the spin adducts measured, because of the large number of adducts trapped. The model made it possible to identify the laser activation of sensitizer molecule in time and its conversion into different free radical products, while the morphological changes of liposomes gave answer on the target of photosensitization which is only a part of the morphological changes observed on the living cells.

G.J. Köteles and Z. Somosy: Are the two other types of erythrocytes (normal biconcave discs, cup-shaped somatocytes) also changing after photosensitization and laser irradiation?

Authors: Yes, however the structural changes observed are not so dramatic as described on the echinocytes, and its demonstration on the SEM pictures is not to remarkable. Please refer to the relevant literature about the morphological changes of different cells on irradiation and about the shape changes of erythrocytes.