Factors Influencing the Haematoporphyrin-sensitized Photoinactivation of *Candida albicans*

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Photosensitizing activity of haematoporphyrin (Hp) on *Candida albicans* cells is mainly promoted by unbound dye molecules in the bulk aqueous medium. Moreover, the death of photosensitized cells is dependent on the dye concentration, irradiation time, irradiation temperature, and the composition of the growth media. Morphological and biochemical studies indicate that the photoprocess involves an initial limited alteration of the cytoplasmic membrane, which allows the penetration of the dye into the cell with consequent photodamage of intracellular targets. In this respect, the Hp-sensitized photoinactivation of eukaryotic microbial cells differs from that in prokaryotic cells.

INTRODUCTION

Irradiation of microbial and mammalian cells with visible light in the presence of oxygen and porphyrins or other photosensitizing dyes (e.g. phenothiazines and xanthenes) promotes a variety of cytotoxic photoprocesses which eventually lead to cell death (Ito, 1983). The biochemical and functional effects of porphyrin photosensitization include cross-linking and photo-oxidation of membrane proteins, peroxidation of lipids, inhibition of transport of some essential metabolites, leakage of lysosomal enzymes and an increased cellular uptake of porphyrins. However, it is not known which types of damage are most important for cell death (Spikes, 1988).

In our laboratory, the photosensitized killing of different microbial cells has been studied in the presence of haematoporphyrin (Hp) (Bertoloni *et al.*, 1984, 1985, 1987). Our results suggest that the progress of the photoprocess is largely influenced by the cellular organization. Thus, for bacterial and mycoplasma cells, the photodamage appears to involve mainly the cytoplasmic membrane, while for yeast cells it involves intracellular loci.

In this paper, we have extended our investigation with yeast cells in order to elucidate the importance of Hp-cell binding for modulating cell photodamage, as well as to define the effect of some experimental parameters (Hp dose, irradiation temperature, composition of the culture media, and phase of cell growth) on the efficiency of Hp-photosensitization.

METHODS

Organism. *Candida albicans* strain O was kindly provided by Dr L. Polonelli, Istituto di Microbiologia, Università Cattolica del Sacro Cuore, Rome, Italy.

Growth media and culture conditions. *C. albicans* was grown in brain heart infusion (Difco) and in Sabouraud broth containing 1% (w/v) proteose peptone, 4% (w/v) glucose, pH 6. The modified media used in this investigation are listed in Table 1. For cell survival assays, these media were solidified with 1.5% (w/v) purified agar. Cells were cultured aerobically at 37 °C.

Abbreviation: Hp, haematoporphyrin.

Table 1. *Media used for cell growth and/or cell survival assay*

Abbreviation	Composition
S ₀	Sabouraud standard, pH 6*
S ₁	S ₀ + NaCl 0.5% (w/v)
S ₂	S ₀ + Na ₂ HPO ₄ 0.25% (w/v)
S ₃	S ₀ + NaCl 0.5% + Na ₂ HPO ₄ 0.25% (w/v)
S - g	S ₀ with glucose concentration reduced to 0.2% (w/v)
S _{7.3}	S ₀ with pH adjusted to 7.3
B ₀	Brain heart infusion, pH 7.3*
B + g	B ₀ with 4% (w/v) glucose
B ₆	B ₀ with pH adjusted to 6.0

* The composition of the standard media is described in Methods. Solid media were obtained by adding purified agar (1.5%, w/v) to the liquid media.

Cell survival assay. Unirradiated and irradiated cells were serially diluted 10-fold in 10 mM-phosphate (Na₂HPO₄ + KH₂PO₄)-buffered saline (PBS), pH 7.2, containing 0.15 M-NaCl; each dilution was plated in duplicate on solid growth medium. After incubation of the plates at 37 °C for 2 d, the number of colony forming units per ml (c.f.u. ml⁻¹) was counted.

Incubation of cells with Hp and irradiation procedure. Cells from cultures in the exponential and stationary phases of growth were harvested by centrifugation at 2000 *g* for 10 min. The sedimented cells were washed twice with PBS by magnetic stirring for 30 min. The cell suspensions used in all experiments had an OD₆₅₀ of 0.87.

Hp, obtained from Porphyrin Products (Logan, Utah, USA), was dissolved in a minimal volume of 0.1 M-NaOH and diluted with PBS to the desired concentration.

The irradiated and unirradiated systems were prepared by adding suitable volumes of the Hp solutions to the cell suspensions, the final dye concentration being in the range 0.1–1000 µg ml⁻¹; the samples were incubated in the dark at room temperature for either 6 or 30 min.

All irradiation experiments were performed using four 250 W tungsten lamps (Osram), which were symmetrically placed on the four sides of the pyrex test tube containing the system to be irradiated (10 ml). The light fluence at the level of the test tube was about 6 mW cm⁻². The cell suspensions were aerated and held at a selected temperature between 2 and 42 °C. To avoid cell sedimentation, the samples were stirred by a small magnetic stirrer during irradiation.

At intervals prior to and during irradiation, samples were taken from the suspension for: (i) cell survival assay; (ii) spectrophotofluorimetric assay; (iii) electron and light microscopy studies; (iv) enzyme activity assay.

Control experiments showed that, under the experimental conditions described above, cells were unaffected by 30 min illumination in the absence of Hp.

Each survival value reported in the figures and tables was obtained by at least five independent experiments.

Light microscopy. For fluorochrome staining of DNA (chromosome), cell pellets were fixed with acetic acid/methanol (1:3, v/v) for 5 min, and centrifuged at 1000 *g* for 5 min; then the fixation was repeated for 10 min. The specimens deposited on slides were air-dried and stained for 30 min at room temperature with 0.05 µg '33258 Hoechst' ml⁻¹ in saline Hanks' solution. The slides were washed three times with twice-distilled water and then covered with a coverslip in the presence of mounting fluid consisting of 50% (v/v) glycerol, 0.02 M-citric acid and 0.05 M-disodium orthophosphate, pH 5.5. Specimens were observed by fluorescence microscopy using no. 50 barrier and BG 12 exciter filters.

In order to estimate the uptake of methylene blue, 0.2 ml volumes of cell suspensions were mixed with 0.05 ml aqueous methylene blue solution (final dye concentration 185 µg ml⁻¹) (Sud & Feinegold, 1981). The cell suspensions were examined microscopically after 5 min exposure to the dye and the number of stained and unstained cells counted. For each experimental condition 1000 cells were considered.

Electron microscopy. Cell suspensions obtained as described above were centrifuged at 3000 *g* for 10 min and the pellets fixed in 3% (v/v) glutaraldehyde in 0.1 M-phosphate buffer, pH 7.2, for 2 h at 4 °C, washed, post-fixed in 1% (w/v) osmium tetroxide in the same buffer for 90 min at 4 °C and washed again. The fixed cells were dehydrated through a graded series of alcohols and embedded in Dow epoxy resin.

The spheroplasts, obtained by enzymic treatment as previously described (Poulter *et al.*, 1981), were fixed by adding commercial glutaraldehyde (Serva) (final concentration 3%, v/v) to the buffer containing 1 M-sorbitol, 4 mM-potassium phosphate, pH 7.0, and Zymolyase 20 T (Miles) 1 mg ml⁻¹, and then treated as for intact cells.

Thin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) and then examined in a Hitachi HS 9 electron microscope.

Estimation of cell-bound haematoporphyrin. The amount of cell-bound Hp was estimated on washed and unwashed cells by a spectrophotofluorimetric procedure (Jori *et al.*, 1986). The spectra were recorded using a Perkin Elmer MFP 4 apparatus, equipped with a thermostatically controlled cell holder; the sample solutions, in 2% (w/v) SDS, were excited at 400 nm and the fluorescence emission was recorded in the 550–750 nm range. The integrated area of the spectra was converted into values of Hp by interpolation with a calibration plot.

The fluorescence emission polarization of cell-bound haematoporphyrin was obtained by inserting a set of polarizers (Hitachi) in both the exciting and emitted beams and recording the 400 nm-excited fluorescence with the polarizers placed in either vertical (90, 0 or 0, 90) or parallel (0, 0 or 90, 90) positions; the degree of polarization, P , at each wavelength was estimated by measuring the fluorescence intensity, I , according to the following equation (Azumi & McGlynn, 1962):

$$P = \frac{I_{0,0} - I_{0,90} (I_{90,90}/I_{90,0})}{I_{0,0} + I_{0,90} (I_{90,90}/I_{90,0})}$$

Assay of enzyme activity. ATPase (EC 3.6.1.3) activity and cytochrome *c* oxidase (EC 1.9.3.1) activity were measured spectrophotometrically as described by Marriot (1975) and Gläser & Höfer (1986), respectively.

RESULTS

Effect of haematoporphyrin dose on cell survival

The survival of *C. albicans* cells, in the exponential and stationary phase of growth, was unaffected after 30 min incubation in the dark with Hp concentrations ranging between 0.1 and 1000 $\mu\text{g ml}^{-1}$.

Clearly, the extent of survival of *C. albicans* in the exponential phase of growth in the presence of Hp at concentrations of 0.1, 1 and 10 $\mu\text{g ml}^{-1}$ was strongly dependent on the dye concentration and illumination time (Fig. 1). In particular, the survival curves obtained upon irradiation with 1 $\mu\text{g Hp ml}^{-1}$ exhibited an initial shoulder, while the time-course of cell death showed a typical exponential decay in the presence of 10 $\mu\text{g Hp ml}^{-1}$.

On the basis of these data all subsequent studies of photosensitized cell inactivation were performed in the presence of 1 $\mu\text{g Hp ml}^{-1}$. Under these conditions, the decrease in cell survival induced by 5 min irradiation was accompanied by a drastic decrease of the cytochrome *c* oxidase activity, while ATPase activity showed a limited decrease (20%) after 1 min irradiation and was

Fig. 1. Survival curves of *C. albicans* cells, obtained from brain heart broth, exposed to visible light at 37 °C in the presence of 0.1 (○), 1 (□) and 10 (△) $\mu\text{g Hp ml}^{-1}$ and plated on brain heart agar. Vertical bars indicate standard error.

Fig. 2. Survival curves of *C. albicans* cells obtained from brain heart broth, irradiated at different temperatures in the presence of $1 \mu\text{g Hp ml}^{-1}$ and plated on Sabouraud agar (a) and brain heart agar (b). Vertical bars indicate standard error.

Table 2. Kinetic studies of enzymic activities of *C. albicans* cells treated with $1 \mu\text{g Hp ml}^{-1}$ in the dark and exposed to light at 37°C

Irradiation time (min)	Percentage of control enzymic activity*		Protein (mg ml^{-1})
	ATPase	Cyt. c oxidase	
10 (no Hp)	100	100	0.82
0	102	105	0.85
1	78.8	96.5	0.79
5	73.2	18.5	0.77
10	70.6	5	0.85

* Enzymic activities are expressed as percentages of the control value obtained with cells irradiated for 10 min in the absence of Hp. The data are the means of two independent irradiation experiments, the maximum deviation from the reported values being within 5% of the mean.

not further affected by prolonged irradiation (see Table 2). These enzymes were chosen as markers of functional properties of the cytoplasmic membrane and mitochondria, respectively.

Effect of the irradiation temperature on cell photosensitivity

As shown in Fig. 2, the cell photosensitivity increased with increasing irradiation temperature. An identical behaviour was observed upon irradiation of cells in the stationary phase of growth under the same experimental conditions, although the overall photosensitivity was slightly reduced. The phenomenon is possibly related to a temperature-induced enhancement of the membrane fluidity; as one can see in Fig. 3, the degree of polarization of the

Fig. 3. Fluorescence polarization spectra measured on *C. albicans* cells treated with $1 \mu\text{g Hp ml}^{-1}$ for 6 min in the dark at temperatures of 9°C (■), 27°C (●), and 37°C (▲).

fluorescence emitted by cell-bound Hp decreased upon increasing the temperature. Such behaviour indicates a greater mobility of the small amount of cell-bound Hp which is largely associated with the cytoplasmic membrane (Bertoloni *et al.*, 1984). On the other hand, the fluorescence emitted by Hp dissolved in homogeneous solutions is essentially devoid of polarization (Srivastava *et al.*, 1973).

Effect of the medium composition on cell photosensitivity

The survival of irradiated cells was dependent on the composition of the medium; in particular, for cells obtained from brain heart broth the survival measured on Sabouraud agar (Fig. 2a) was constantly greater than that measured on brain heart agar (Fig. 2b). The same trend, with a slight increase in survival, was observed for cells obtained from Sabouraud broth and plated on both solid media.

The Hp-binding capacity of cells was not modified by varying the composition of the growth medium. These data were obtained with cells cultured in S_0 , S_1 , S_2 and S_3 , in which the amount of tightly bound Hp was in the 1.6%–2.0% range. This is in agreement with previous data obtained with cells cultured in brain heart broth (Bertoloni *et al.*, 1987).

The presence of salts in the plating media did not change the percentage of cell survival upon 10 min irradiation. However, survival was greater for cells obtained from salt-free or phosphate-containing Sabouraud, than for cells obtained from corresponding NaCl-containing media (Fig. 4a). Similarly, glucose added to brain heart agar enhanced the survival of irradiated cells, while the presence of even reduced concentrations of glucose in Sabouraud did not alter the survival (Fig. 4b). Finally, the pH value affects the survival of only those irradiated cells obtained from Sabouraud medium at pH 6 (Fig. 4c).

The pH of the irradiation medium exerted no significant effect on cell survival, at least in the range examined (pH 5–8).

As shown in Fig. 5, cell survival decreased upon increasing the PBS concentration in the irradiation medium.

Effect of bound Hp on cell photosensitivity

Determinations of the amount of cell-associated porphyrin after repeated washings with PBS indicate that at least 98% of Hp is not tightly bound to *C. albicans* (Bertoloni *et al.*, 1987). Survival of thrice-washed cells (which retain only tightly bound Hp) decreased only after prolonged light exposure (Table 3). The washed cells harvested during irradiation and plated after 120 min dark incubation underwent a further decrease in survival (Table 3). Decrease in the survival of washed and irradiated cells was accompanied by a gradual increase in cell permeability to methylene blue (Table 3). A similar process was observed in the case of unwashed and irradiated cells (Bertoloni *et al.*, 1987); however, the enhancement of cell permeability was much faster, reaching 100% after 7 min irradiation.

Fig. 4

Fig. 5

Fig. 4. Effect of the composition of culture and plating media (*a*, salts; *b*, glucose; *c*, pH) on the survival of *C. albicans* cells irradiated at 37 °C for 10 min in the presence of 1 µg Hp ml⁻¹. The abbreviations used for the media are the same as specified in Table 1. The abbreviations on the abscissa indicate the liquid growth media used, while the abbreviations in the plot indicate the media used for cell survival assay.

Fig. 5. Effect of the PBS concentration in the irradiation medium on the survival of *C. albicans* cells, obtained from brain heart broth, irradiated at 37 °C in the presence of 1 µg Hp ml⁻¹ and plated on brain heart agar. The number at the right end of each plot indicates the ratio between the PBS concentration (phosphate + NaCl) used and the standard PBS concentration (10 mM-phosphate + 0.15 M-NaCl).

Table 3. *Kinetic studies of survival and membrane permeability of C. albicans cells treated with 1 µg Hp ml⁻¹ for 6 min in the dark, washed three times with PBS and exposed to light at 37 °C*

Irradiation time (min)	Percentage survival of irradiated cells		Percentage uptake of methylene blue determined immediately after light exposure*
	Plated immediately	Plated after 120 min dark incubation	
0	100	100	2
10	81	59	2
30	7.3	4.5	14
60	0.13	0.02	40
90	0.013	0.005	63
120	0.0014	0.0004	78

* For each experimental condition 1000 cells were considered.

Light and electron microscopy

Hp-photosensitized cells exhibited drastic alterations of their morphology. In particular, cells irradiated for 10 min were characterized by decreased electron-density and the appearance of intracellular vacuolization (Fig. 6*a-c*). These alterations are more evident in the micrographs of the spheroplasts obtained from unirradiated and irradiated cells. Spheroplasts obtained from

Fig. 6. Electron micrographs of *C. albicans* cells (*a–c*) and spheroplasts (*d–f*). (*a,d*) Cells exposed to light for 10 min in the absence of Hp. (*b, c, e, f*) Cells treated with 1 µg Hp ml⁻¹ for 6 min in the dark and exposed to light for 1 min (*b, e*) or 10 min (*c, f*). Bars, 1 µm.

control samples appear as rounded and lobed cells, surrounded by a well-defined cytoplasmic membrane (Fig. 6*d–f*). After 1 min of exposure to Hp plus light, the cells became less electron-dense, with a spheroidal shape and a ragged membrane (Fig. 6*e*). Upon increasing the irradiation time (10 min) the cytoplasmic membrane was broken and most intracellular organelles were no longer identifiable (Fig. 6*f*).

Fig. 7. Fluorochrome staining of *C. albicans* cells. (a) Cells exposed to light for 30 min in the absence of Hp. (b-d) Cells treated with $1 \mu\text{g Hp ml}^{-1}$ for 6 min in the dark and exposed to light for 1 min (b), 10 min (c) or 30 min (d). Bars, $10 \mu\text{m}$.

These morphological modifications were accompanied by a different interaction of cells with the fluorochrome bis-benzimide, which stains the cellular DNA (Fig. 7). In the control cells irradiated without Hp, and in cells irradiated for 1 min in the presence of $1 \mu\text{g Hp ml}^{-1}$, the fluorescence was localized in the central part which was spheroidal in shape. In the cells irradiated for 10 min the extent of the fluorescent area was reduced, whilst in the cells irradiated for 30 min a spreading of the fluorescence was observed.

DISCUSSION

Our results show that only concentrations of Hp above $1 \mu\text{g ml}^{-1}$ in the incubation medium induce a significant decrease of the survival of cells irradiated for 10 min; it is likely that any photodamage caused by lower concentrations of Hp occurs at a sufficiently low rate to allow its repair (Moan & Sommer, 1985). This hypothesis is supported by the lack of detectable cell death in the presence of $0.1 \mu\text{g Hp ml}^{-1}$ and by the shoulder observed in the survival curves of *C. albicans* at $1 \mu\text{g Hp ml}^{-1}$ (see Fig. 1); this feature disappears when higher Hp concentrations are used.

Moan & Stenstrom (1981) observed that the Hp-photosensitized inactivation of *Saccharomyces cerevisiae* was enhanced by the presence of phosphate or chloride ions in the irradiation medium. Our data indicate that the presence of these salts in the media used for cell growth and/or cell survival assay influences the photodynamic inactivation of *C. albicans* cells. Moan & Stenstrom (1981) suggested that this effect may be the consequence of either a dependency of the amount of cell-bound Hp on the concentration of these ions or an interference of phosphate and chloride ions with the processes which repair the photoinduced cell damage. In our experiments the concentration of Hp bound to *C. albicans* cells was essentially independent of the nature of the medium, indicating that processes which counteract the effect of Hp photosensitization are important. The different extent of cell survival could be due to partial repair of the photodamage, for cells plated immediately after irradiation, or its continuation during dark incubation.

Interestingly, in the system studied by us, Hp carries out its photosensitizing action almost exclusively from the bulk aqueous medium. Only a small fraction of added Hp (less than 2%) becomes tightly bound to cells and is not removed by repeated washings (Bertoloni *et al.*, 1987). This agrees with previous results obtained by Ito (1981) on the porphyrin-photosensitized inactivation of yeast cells. However, binding of Hp to cells is essential for efficient photosensitization of Mollicutes and Gram-positive bacteria (Bertoloni *et al.*, 1984, 1985). These observations emphasize the importance of the nature of the microbial cells and the subcellular localization of the sensitizer in determining the mechanism of Hp-photosensitization. Thus, for both mycoplasmas and bacteria, the cytoplasmic membrane represents the main site of cell photodamage, in agreement with the large amounts of Hp accumulated by this cell structure (Bertoloni *et al.*, 1984, 1985). In the case of *C. albicans*, Hp-photosensitization apparently causes only minor damage to the cytoplasmic membrane. The ATPase activity of irradiated cells is impaired by about 20% after 1 min and undergoes no further decrease up to 10 min. Moreover, the electrophoretic pattern of cytoplasmic membrane proteins of extensively irradiated cells shows a small extent of cross-linking (Bertoloni *et al.*, 1987). These modifications may affect membrane properties, as shown by the appearance of rounded cells in the electron microscopy studies. The latter process may allow the penetration of originally external Hp molecules into the cells. This hypothesis is supported by the observed kinetics of methylene blue uptake after irradiation of washed cells.

The survival kinetics obtained with cells irradiated in the presence of different PBS concentrations suggest that an appropriate osmolality of the irradiation medium can reduce the killing effect. The osmolality of the irradiation medium and the fluidity of the cytoplasmic membrane could interfere with the survival rate simply by modulating the penetration of the dye into the cells. Actually, an increase of membrane fluidity causes an enhanced efficiency of photosensitized cell death (see Figs 2 and 3). The above consideration suggests that the main photodamage occurs at an intracellular level. In particular, we observed a marked drop in the cytochrome *c* oxidase activity, as well as the formation of large vacuoles, and alterations of the architecture of intracellular organelles. It is likely that the vacuolization causes compression of the nuclear material, thus explaining the initial compression of DNA as seen by fluorochrome staining; the fluorescence spreading (Fig. 7) is indicative of damage to the nuclear envelope.

In conclusion, our data indicate that the Hp-photosensitization of *C. albicans* cells causes a limited initial damage of the cytoplasmic membrane. Such damage probably permits the penetration of photosensitizer molecules into the cytoplasm, leading to the damage of targets which are critical for cell survival.

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