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In Vitro Cellular Effects of Hematoporphyrin Derivative[1]

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ABSTRACT

Several *in vitro* cell systems were exposed to hematoporphyrin derivative (HPD): established lines of rat kangaroo epithelial kidney; normal mouse embryonic fibroblasts; and differentiated neonatal rat myocardial cells. The uptake of HPD (25 to 100 μg/ml) by individual cells occurred rapidly over a 2-hr period and leveled off by 24 hr. HPD was excreted from cells by 48 hr after exposure. However, a low level of HPD (above background) was maintained in cells for up to 4 days following cessation of exposure. Intracellular binding of HPD was to mitochondria as demonstrated by fluorescence microscopy. HPD was also shown to have a growth-inhibiting effect on rat kangaroo cells without added light. The growth effects on mouse cells were less marked.

INTRODUCTION

The use of HPD[3] plus light as an effective modality in the management of cancer is currently receiving considerable attention (5, 7-9).

Even though the method appears to have strong potential as a therapeutic modality, many questions are still unanswered. The active ingredients in the HPD solution have not been determined. In addition, the question of whether or not binding of the HPD and/or its individual subfractions is to the cell surface and/or to various regions and organelles inside the cell has not been resolved (3, 4, 10, 12, 16, 17). Furthermore, the precise mode of cytotoxicity and the mechanism of selective retention by tumor tissue are not understood (2, 6, 13, 17, 18).

We have approached the above questions using *in vitro* as well as *in vivo* models. In the present study, we have investigated HPD effects on normal cells. It was felt that, before examining the effect of combined HPD plus light on malignant cells, the effects of HPD on normal cells should be examined. This question seemed particularly important since HPD is presumed to have few or no deleterious effects on normal tissues, despite the fact that a patient given injections of 3 to 5 mg of HPD per kg body weight will have a strong hypersensitivity to normal daylight for up to 1 month following HPD injection.

MATERIALS AND METHODS

HPD. HPD was obtained from Dr. Thomas Dougherty, Roswell Park Memorial Institute. Vials containing 5 mg HPD per ml of 0.9% NaCl solution were stored in the dark until used. All cell treatments reported in this paper used 25 to 100 μg of HPD per 100 ml of culture medium containing 10% fetal calf serum.

Cells. One cell line used was the established rat kangaroo cell line PTK_2 (ATCC No. 56; American Type Culture Collection, Rockville, Md.). These are epithelial cells originally derived from the kidney of a male rat kangaroo *Potorous tridactylus*. The line has a near-diploid condition of $2n + 1$. The cells are contact inhibited and do not exhibit malignant morphological features. They also remain flat during mitosis, thus making detailed cytological observation particularly easy.

A second cell line used was the established line BALB/3T3 clone A31 (ATCC No. CCL 163). This cell line was chosen because it is a more commonly used fibroblast line and there are variant lines available that exhibit malignant characteristics (most notably the BALB/#T12-3 line).

Cells are grown either in T25 plastic flasks for growth studies (14) or on glass coverslips in multipurpose Rose tissue culture chambers for fine-resolution fluorescent and light microscopy.

Primary cultures of neonatal rat myocardial cells were also used. These cells were established according to standard procedures from 1- to 3-day-old rat ventricles (11). These cells maintain normal morphology and contractile behavior for up to 1 month in culture.

Growth Studies. Standard growth curves were run on PTK_2 and 3T3 cells grown in T25 flasks. The PTK_2 cells were seeded into flasks at 50,000 cells/flask. The following day, fresh medium with 25 μg of HPD per ml was added, and the cells were exposed for 6, 8, 18, or 24 hr. The 3T3 cells were seeded at 10^5 cells/flask. The following day, fresh medium with 100 μg of HPD per ml was added, and the cells were exposed for 6 or 24 hr.

With both cell lines, the medium was changed daily for the first 4 days following drug exposure. For the remainder of the experiment, the medium was changed every other day. Two flasks were harvested by enzymatic treatment from each group at each medium change, and the viable cells were counted on a Model Zbi Coulter Counter (Coulter Electronics Inc., Hialeah, Fla.).

All cell handling was performed in near darkness, and the cells were maintained in a 37°, 5% CO_2 incubator in the dark. The PTK_2 cells were grown in minimum essential medium (Eagle's) with Earle's salts supplemented with sodium pyruvate (0.11 g/liter) and 10% fetal calf serum. The 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Media were obtained from Grand Island Biological Co., Grand Island, N. Y. Sera were obtained from Flow Laboratories, Rockville, Md.

Fluorescence Analysis. Intracellular fluorescence emission spectra were obtained using a Nanospec 10S microspectrofluorometer (Nanometrics, Inc., Sunnyvale, Calif.) mounted above a Zeiss RA epifluorescence microscope equipped with a 100-watt mercury lamp and excitation-barrier filter set No. 487709 (Carl Zeiss, Inc., New York, N. Y.). Fluorescence of individual single cells were recorded by positioning them under an aperture in the optical system of the microscope. Emitted fluorescence was projected into the aperture and subsequently to the photomultiplier (1).

Individual fluorescent measurements were also made on PTK_2 cells using a laser-excited fluorescence system (15). This system involves focusing a 442-nm helium-cadmium laser beam to a microspot of 0.5 to 20 μm in diameter inside a single cell. The fluorescence is detected by a sensitive EMI No. 9862B photon-counting photomultiplier (EMI Gencom, Inc., Plainview, N. Y.) and accumulated by a Tracor Northern

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[3] The abbreviation used is: HPD, hematoporphyrin derivative.

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No. 1710 multichannel analyzer (Tracor Northern, Inc., Middleton, Wis.). With this system, accurate fluorescent measurements were made on individual cells. This system was also used to record the changes in fluorescence in the mitochondria of single heart cells that had been treated with both HPD and the respiratory-sensitive fluorochrome Rhodamine 6G (15). Three- to 7-day-old cultures were stained with the fluorescent probe Rhodamine 6G (1.0 μg/ml culture medium) for 30 min at 37°. The cells were rinsed in three 10-min washes of fresh culture medium and then observed for fluorescence by focusing a helium-cadmium laser beam (λ 442 nm) through a Zeiss RA microscope to a 0.5-μm spot.

Another system using a Tracor Northern No. 1223-3GI diode array was used to study the spatial distribution of HPD fluorescence across an individual cell. This system involved the projection of the fluorescence image of a cross-section through the cell onto an array of 1024 small diodes.

RESULTS

The fluorescent emission spectra from within single cells were different from the spectra of noncellular bound HPD. A drop of HPD in 0.9% NaCl solution on a slide exhibited 3 distinct peaks at 610, 640, and 675 nm, whereas HPD in 0.9% NaCl solution on a piece of filter paper exhibited a distinct red shift of fluorescence with peaks at 620, 645, and 685 nm. The emission spectrum of HPD bound in the cells was blue shifted by 10 to 20 nm with 3 peaks at 600, 635, and 670 nm. In addition, the fluorescent peak with the shortest wavelength was always relatively lower in intensity than were the 2 longer wavelength peaks in the cells, whereas in the noncellular preparation the first peak was always higher in intensity than were the 2 longer wavelength peaks (Chart 1).

Quantitative Cellular Fluorescence. Fluorescence readings were made on individual cells using the laser-stimulated fluorescence detection system. HPD uptake was determined by measuring fluorescence at 15- to 20-min intervals over the first 2 hr following addition of HPD (25 μg/ml) to the culture medium (Chart 2). There is a rapid uptake over the first 2 hr and a gradual leveling off by 24 hr.

In a subsequent experiment, the excretion of HPD was measured starting at 24 hr after the removal of the HPD-containing solution. The cells treated with HPD for 18 hr maintained a high level of HPD through 24 hr posttreatment (Chart 3). By 48 hr posttreatment, a substantial loss of HPD from the cells had occurred. The amount of HPD in the cells decreased only slightly over the next 48 hr, and there appeared to be a low level (above control measurements) of HPD maintained within the cells.

Intracellular Localization of HPD. Fluorescent microscopy

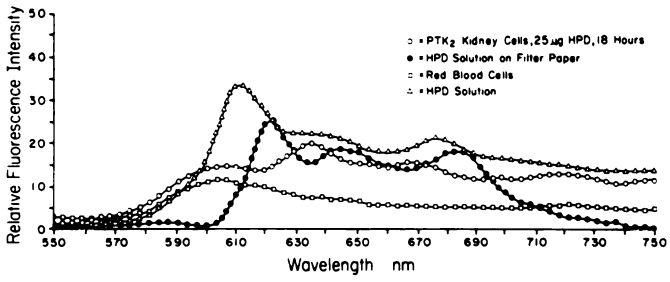

Chart 1. Fluorescence emission spectra of HPD in cells and 0.9% NaCl solution; porphyrin of normal RBC.

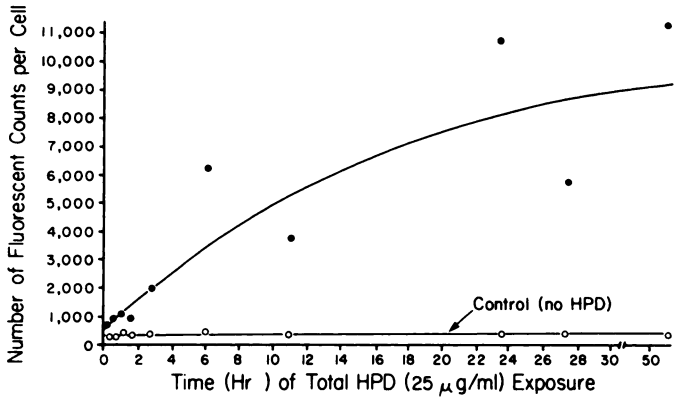

Chart 2. Uptake of HPD by tissue culture cells as measured by fluorescence measurements on individual cells. *Points*, mean of at least 6 different measurements from different cells.

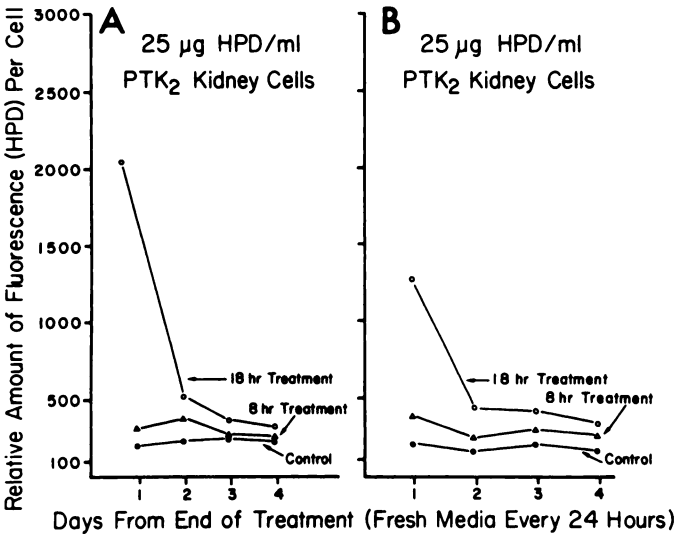

Chart 3. Replicate experiment of loss of HPD from cells. *Points*, mean of at least 6 separate measurements in different cells.

revealed a distinct orange fluorescence in the cytoplasm of the PTK_2 cell with the greatest intensity of fluorescence around the outside of the nucleus. Furthermore, a quantitative reading of fluorescence across the cell with the diode array (Chart 4) demonstrated a distinct perinuclear fluorescence pattern. This pattern correlates with the distribution of lysosomes and mitochondria in these cells. The diode array fluorescence pattern is reflecting a real distribution of the organelle-associated HPD in both the horizontal plane and depth.

The effect of HPD on mitochondria was further examined by recording the fluorescence patterns from individual heart cell mitochondria that had been exposed to the fluorescent probe Rhodamine 6G and HPD. It has been shown previously that Rhodamine-treated mitochondria exhibit oscillating fluorescence (15) and that this fluorescence can be blocked by specific inhibitors of the electron transport chain. Chart 5*A* illustrates a normal oscillating fluorescence pattern of a Rhodamine 6G-stained mitochondrion. Chart 5*B* is a nonoscillating fluorescence pattern of a mitochondrion in an HPD-treated cell at 12 min following HPD treatment. This inhibition is similar to that found for metabolic inhibitors (15). Furthermore, since the measurements are made from the cell side directly apposed to

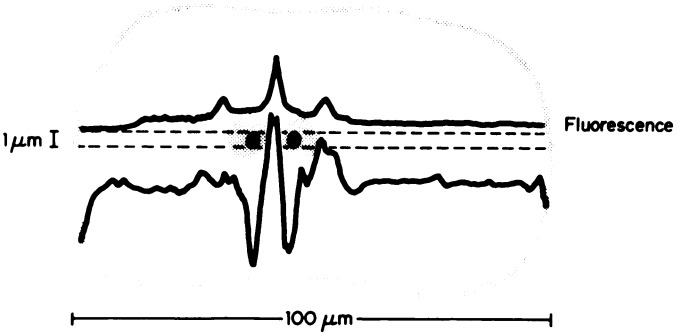

Chart 4. Intracellular distribution of HPD fluorescence. *Outer gray line*, membrane of cell. Two *small dots* in center are 2 nucleoli each in a separate nucleus (*gray circular objects*). The entire cell was exposed to a fluorescence-stimulating mercury lamp. A 1-μm-wide x 100-μm-long section (the region between the 2 *dashed lines*) through the fluorescing cell was projected onto a diode array. *Upper dark line*, relative fluorescence across the cell; *peaks*, increased fluorescence intensity; *lower dark line*, indication of the contrast difference through the same region. A phase-contrast image of the same cell region was also projected onto the diode array. It was therefore possible to match fluorescence with known phase-contrast objects.

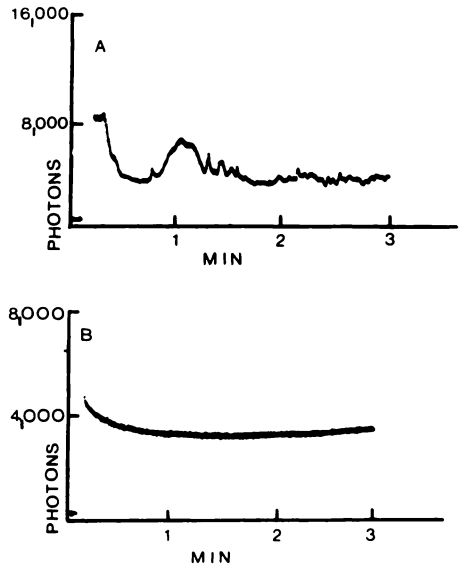

Chart 5. *A*, fluorescence emission at a fixed wavelength of a 1-μm region of a single mitochondrion stained with Rhodamine 6G. Note the oscillating fluorescence pattern. *B*, a similar emission taken from a mitochondrion stained with Rhodamine 6G and also treated with HPD (25 μg/ml) for 20 min. Note the decrease in fluorescence and the lack of the oscillating patterns observed in *A*.

the glass coverslip, no interference from overlying media is possible.

An examination of heart cells treated only with HPD revealed distinct orange fluorescence localized specifically to the large mitochondria (Fig. 1).

Cell Survival. PTK_2 cells treated with HPD (25 μg/ml) for 18 hr exhibited a definite reduction in growth rate. Most of the cells exposed to HPD (25 μg/ml) for 24 hr were killed by Day 9 posttreatment (Chart 6*A*). 3T3 cells treated with HPD (100 μg/ml) demonstrated a reduction in cell number at 18 to 24 hr posttreatment but resumed log-phase growth by 36 to 48 hr posttreatment (Chart 6*B*).

DISCUSSION

Earlier studies on HPD in solution demonstrated 2 distinct fluorescence peaks, one at 615 to 635 nm and the other at

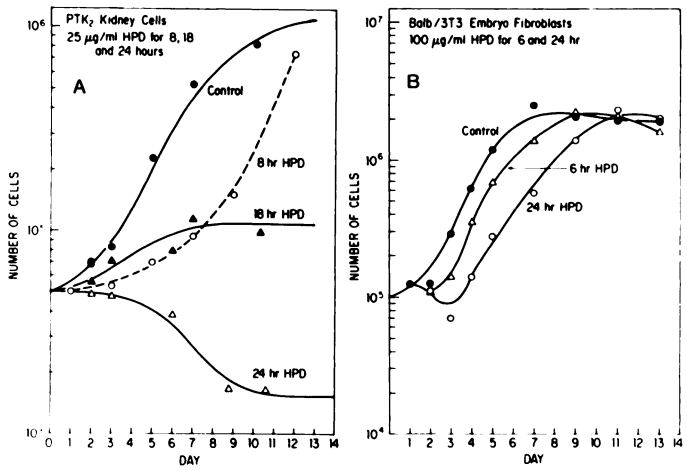

Chart 6. Growth curves of cells following treatment with HPD. *A*, PTK_2 cells treated with 25 μg/ml at Day 1 for 8, 18, and 24 hr; *B*, 3T3 cells treated with 100 μg/ml for 6 and 24 hr starting at Day 1. Note that the 6-hr cells had resumed log-phase growth by Day 3 (30 hr postcessation of exposure) and the 24-hr cells had resumed log-phase growth by Day 4 (48 hr postcessation of exposure).

580 to 590 nm.[4] However, when measured on the more sensitive microfluorometer used in this study, the longer wavelength fluorescence emission could be resolved into 3 distinct peaks. These peaks demonstrated a blue shift when measurements were taken from inside single cells. Furthermore, the relative proportion of the 3 peaks was altered when the HPD was bound inside the cell. These shifts and changes in intensity may reflect the binding and localization of specific subfractions of HPD.

The quantitative measurements on the cells demonstrated a rapid uptake of HPD (or one of its components) by 2 hr of treatment. Significant amounts of a porphyrin component were detected in the cells within 10 min of treatment indicating a rapid internalization. The fluorescence microscope also revealed an intracellular pattern of fluorescence surrounding the nucleus. Little or no fluorescence was detected at or near the cell surface or inside the nucleus. This distribution pattern was confirmed by the diode array measurements across the cell.

The studies on the mitochondrial fluorescence patterns demonstrated that HPD rapidly inhibits fluorescence oscillations that are detected by monitoring Rhodamine 6G fluorescence in single mitochondria. Direct observation with the fluorescence microscope confirms that HPD fluorescence is very intense in the large mitochondria of cardiac cells. These observations further substantiate the suggestion of other investigators (12) that the mitochondria are affected by the internalized fraction of HPD.

Quantitative HPD determinations on cells that have been removed from the HPD-containing culture medium demonstrate a loss of HPD from cells by 40 hr post HPD treatment. However, a low level of HPD is retained in cells for up to 4 days posttreatment. Measurements were not made past this time period but, by the slope of the excretion curve, it is likely that a low level of HPD will be maintained in these cells for a considerable period of time.

Growth studies on HPD-treated PTK_2 cells clearly demonstrate that concentrations of HPD at 25 μg/ml for 18 and 24 hr

[4] M. W. Berns, unpublished observations.

have a growth-inhibiting and toxicity effect even though the cells were not exposed to light.

The results with the 3T3 cells indicate an initial growth-inhibiting effect of HPD but no long-term negative effect. The differing results for the PTK_2 and 3T3 cells suggest that HPD may affect different cells in various ways. This may explain why other investigators have not detected a cytotoxic effect of HPD alone. In addition, these results may not have been detected by other investigators because their cells were simply not followed over a long enough time period or because control studies using HPD-treated, nonirradiated cultures were not tested.

The data suggest that HPD definitely affects normal cells without any light addition. It is also evident that, even though HPD does come out of normal cells rather rapidly, a small amount is retained. We have yet to determine if the retained HPD confers a photosensitization to the cells. Studies are under way comparing normal and malignant cells.

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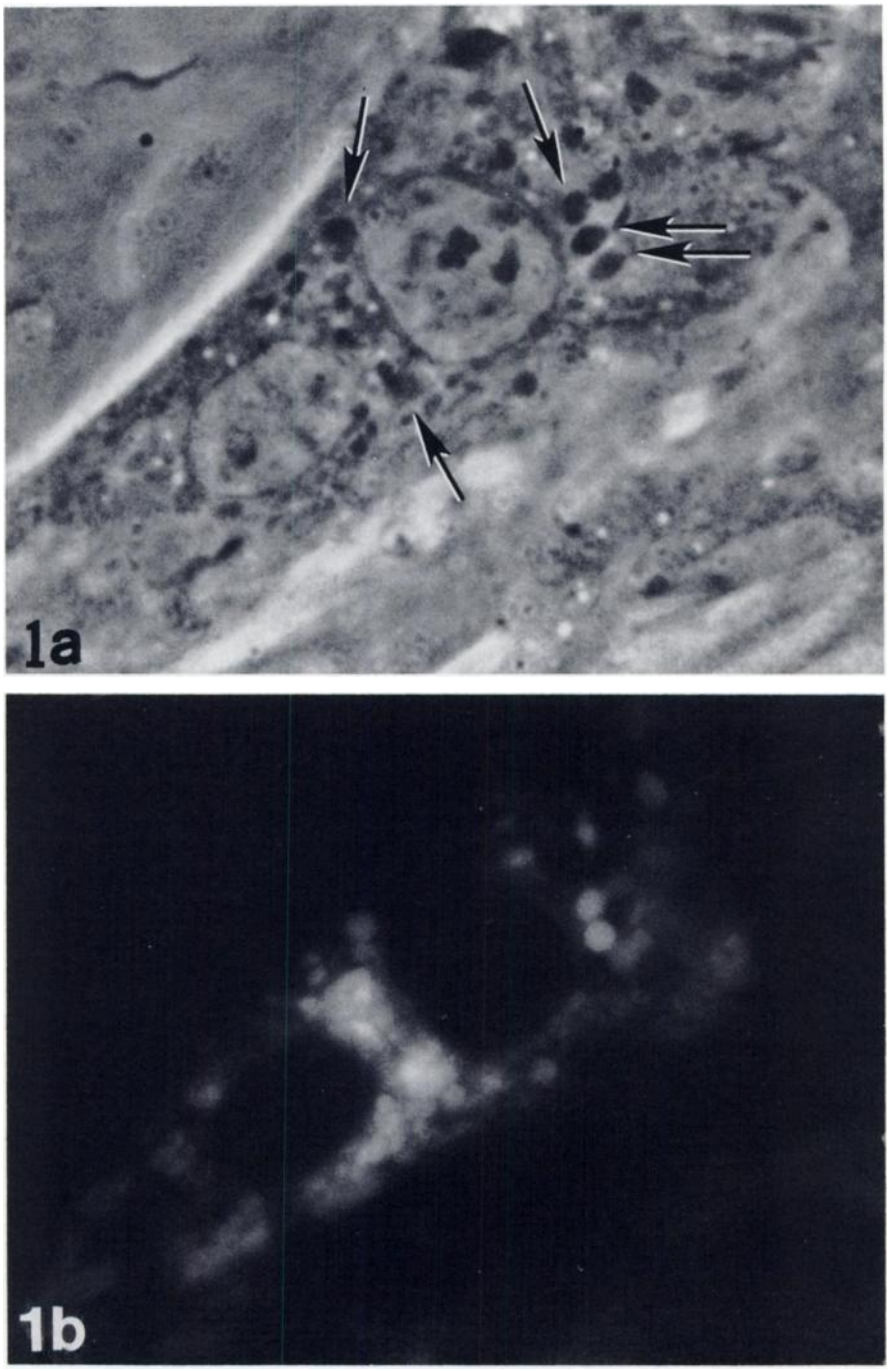

Fig. 1. *a*, live phase-contrast micrograph of rat myocardial cell. Phase dark bodies (*arrows*) are mitochondria. × 1400. *b*, fluorescent micrograph of same cell after treatment with HPD (25 μg/ml) for 1 hr. Note the bright fluorescence confined primarily to the mitochondria. × 1400.