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Photodynamic Killing of Retinoblastoma Cells with Hematoporphyrin and Light[1]

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ABSTRACT

Hematoporphyrin and white light exerted a lethal effect on two cell lines of retinoblastoma cells. The lowest values of dye concentration and light exposure capable of killing an entire cell population were, respectively, 2×10^{-5} M and 6 min exposure to an irradiance of 6.0 microwatts/sq mm. Cells exposed to light in the presence of the dye did not require lengthy incubation periods but were rapidly killed with increasing periods of light exposure. Cells washed free of the dye, however, required a minimum sensitizing period of 3.5 hr to achieve a value close to 100% cell death. An inhibitor of this photodynamic process was demonstrated in normal serum. When the concentration of either fetal calf serum, rabbit serum, or rabbit plasma was increased to 25% from a standard 10%, there was as much as a 100-fold greater requirement of hematoporphyrin concentration to produce the same lethal response. The suggested explanation for this phenomenon is a porphyrin-binding plasma factor, hemopexin, which is a natural β-glycoprotein believed to be responsible for the transport of circulating porphyrins to the liver for their elimination. Retinoblastoma cells grow in suspension and thereby provide an excellent tool for study of photosensitive dyes, especially in the case of the rapidly growing Y79 cell line.

INTRODUCTION

Chemical compounds which can be selectively concentrated by neoplastic tissues are of special importance in the field of cancer chemotherapy. Certain porphyrin derivatives, long known to possess this property, have for half a century been of considerable interest for diagnostic purposes. Policard (18) first recognized hematoporphyrin for its diagnostic value in human and animal tumors. He noted that tumors fluoresced selectively to UV after i.v. injection of the dye. Others (6, 9, 10–12, 15, 17, 20, 25, 27–30) later demonstrated the same response for a number of natural and synthetic porphyrins. The possible therapeutic use of hematoporphyrin was anticipated by a number of authors, as reviewed by Tsutsui *et al.* (25), but only in the last few years has this value been fully appreciated. Diamond *et al.* (1) and Granelli *et al.* (7) demonstrated the cytotoxic action of hematoporphyrin and white light against rat gliomas *in vitro* and *in vivo*. Dougherty *et al.* (5) were able to obtain a significant rate of long-term cures in several mouse and rat carcinomas, accomplished by a properly scheduled timing and dose of hematoporphyrin in conjunction with irradiation with red light. More recently, Dougherty *et al.* (3) used hematoporphyrin to treat 9 patients with different types of cancer. The neoplasms included chest wall metastases following breast surgery, abdominal masses following colorectal surgery, malignant melanoma, squamous cell carcinoma, and basal cell carcinoma. All tumors treated show a marked and rapid response. Another recent study on human cancer was performed by Kelly and Snell (8), who were able to produce tumor damage in bladder carcinoma but only to malignant sites within reach of a catheterized light probe.

The cytotoxic action of hematoporphyrin and light is probably due to the formation within the cells of singlet oxygen, a short-lived, highly reactive state of the oxygen molecule (4, 26).

Retinoblastoma, a cancer of photoreceptor elements of the retina, is the most common intraocular cancer in children (23, 24). Its presence is not readily apparent in infancy but is usually discovered before the age of 2. Although growth of some retinoblastomas can be arrested by photocoagulation, X-irradiation, cryotherapy, or chemotherapy, many are controllable only by enucleation of the eye. Often, bilateral enucleation becomes necessary. The possible use of phototherapy, with hematoporphyrin derivatives as tissue sensitizers, has special significance for ocular cancer because the ocular media are transparent and the sclera and underlying structures are thin enough to transmit light.

In the present report, 2 recently established retinoblastoma cell lines (13, 19) are shown to be highly sensitive to irradiation by white light after sensitization with μg quantities of hematoporphyrin.

MATERIALS AND METHODS

Retinoblastoma Cell Lines. The Y79 (19) and WERI-Rb1 (13) cell lines were grown in suspension in $RPMI_{10}$[2] and 2.0 mmol L-glutamine (Grand Island Biological Co., Grand Island, N. Y.). The cells were grown in 100-ml medium bottles in an upright position in 25- to 35-ml amounts. Incubation was at 37° in a humidified chamber supplemented with 95% air:5% CO_2. Medium was replaced 1 to 2 times a week, and cell transfers were made once each week at a 1:10 split ratio for Y79 cells and 1:2 or 1:3 for WERI-Rb1 cells.

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[2] The abbreviations used are: $RPMI_{10}$, Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 10% heat-inactivated fetal calf serum; FCS, fetal calf serum.

Hematoporphyrin (M.W., 599) was obtained from the Sigma Chemical Co., St. Louis, Mo., and kept dry at −20° over $CaCl_2$ in a tightly sealed container. Solutions were prepared in semidarkness by mixing 500 mg with 20 ml 0.1 M NaOH in an Erlenmeyer flask and neutralizing after 15 to 30 min with approximately 5 ml 0.1 M HCl. Solutions of hematoporphyrin were stored at 4° in the dark and retained potency for over 90 days.

Light Source. A 15-watt cool white fluorescent bulb was used in an apparatus with a rocking platform (Drummond Scientific Co., Broomall, Pa.). The temperature was maintained at 35–37° by a hair dryer. The distance from light to the platform was 10 cm, and the light irradiance of 6.0 microwatts/sq mm was measured by a radiometer (E. G. & G. Electro-Optics Division, Salem, Mass.) consisting of a Model 450-1 photometer/radiometer and a Model 550-2 multiprobe detector reading values directly in microwatts/sq cm. The multiprobe was fitted with a flat radiometric filter designed to correct the detector response to a constant sensitivity [±7% (S.E.)] over the spectral range from 460 to 975 nm.

Cell Sensitivity to Hematoporphyrin. Retinoblastoma cells were washed and diluted in $RPMI_{10}$ and brought to a cell density of 1 to 2 × 10^6 cells/ml. Serial 2-fold dilutions of hematoporphyrin (in $RPMI_{10}$), in 0.5-ml amounts, were prepared in duplicate in Wasserman test tubes or in similar plastic disposable tubes. These tubes were capable of transmitting all visible wavelengths of light as determined by use of a monochromator and radiometer. Retinoblastoma cells were then added to each tube in 0.5-ml amounts, thereby halving hematoporphyrin values to their final concentrations. One set of tubes was used for light exposure, and the other set was used for dark controls. Another set of control tubes in triplicate, without added hematoporphyrin, determined the effect of light alone. All preparations were carried out with sterile technique and in semidarkness. Initially, hematoporphyrin-containing cell cultures and their controls were incubated overnight at 37°, after which the proper mixtures were exposed to light. Subsequent experiments effectively utilized a 6-hr sensitization period. Once sensitized, the cultures were exposed to the light source for 30 min, after which they were reincubated overnight before being examined for cell viability.

To determine if serum factors were inhibitory to the photodynamic action of hematoporphyrin, the same experiment was performed with FCS at 25% instead of 10%. The dye at 5120 μg/ml was incubated at 37° for 24 hr with an equal volume of FCS and then prepared with 2-fold decreasing dilutions of hematoporphyrin and a constant level of serum at 25%. Y79 cells in Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 25% heat-inactivated FCS were added prior to light exposure.

Following standard cell culture procedures, heat-inactivated serum was used initially in this experiment, but subsequently the process was repeated with non-heat-inactivated serum to determine if the suspected inhibitory factor(s) was a laboratory-induced artifact. The same procedure was repeated with 25% normal rabbit plasma.

Cell Viability. Determinations of cell viability were made separately by vital staining with trypan blue and neutral red at a final concentration of 0.05%. Trypan blue was excluded by living cells but rapidly absorbed into the nucleus of dead cells. Neutral red slowly accumulated, over a 5- to 30-min period, in the lysosomes of living cells but was excluded from the dead cells. Ratios of living to dead cells were obtained by preparing stained wet mounts of each culture and obtaining differential counts within a grid projected by a microscope reticle. A total of 500 cells was counted for each culture, and the S.E. was obtained from 3 to 5 counts made in different regions of the slide. Confirmation of results was accomplished by repeating every experiment at least twice. Vital dye staining, which is normally a very poor method for quantitative determination of cell viability, was found to give accurate and easily reproducible results with retinoblastoma cells because of their normal growth in suspension rather than in a monolayer. With minimum agitation to disrupt living and dead clusters, individual cells were readily distinguishable for counting. The S.E. for most of the measurements fell below ±7%. Only for values near the middle ranges of cell death did the S.E. occasionally go as high as ±18%.

RESULTS

Cell Sensitivity to Hematoporphyrin. The WERI-RB1 and Y79 cell lines were equally sensitive to photoradiation with hematoporphyrin and showed 50% cell death at approximately 5 μg of dye per ml and 6.0 microwatts of irradiance per sq mm for 30 min. Cell death reached 100% at a concentration close to 10 μg/ml (1.67 × 10^{-5} M) as shown in Chart 1. Cultures in the dark control group, in the presence of all levels of hematoporphyrin up to 1280 μg/ml and for periods up to 4 days, remained healthy and viable. Cultures without hematoporphyrin but exposed to light were also normal and showed only a natural background cell death of 5 to 10%. They had typical grape-like clusters of round cells, as well as small chain formations and single cells showing dye exclusion for trypan blue, and neutral red

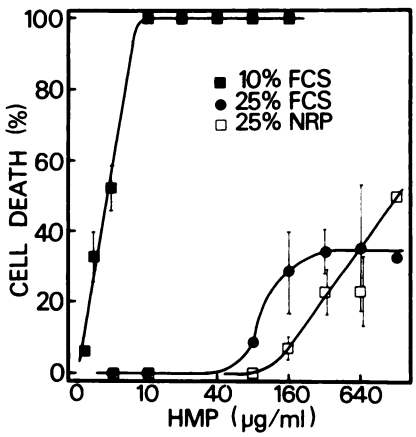

Chart 1. Percentage of cell death (less control value of 7% dead cells initially present) for retinoblastoma cell line Y79 at varying concentrations of hematoporphyrin. Separate *graphs* represent the normal control medium with 10% FCS and the inhibitory influence of higher concentrations of 25% FCS or 25% normal rabbit plasma (NRP). Serum and plasma specimens were heat inactivated. All cultures were sensitized with hematoporphyrin for 6 hr at 37° prior to 30 min exposure to a 15-watt cool white fluorescent light, at a distance of 10 cm, and received an irradiance of 6.0 microwatts/sq mm. *Bars*, S.E. for experiments run in triplicate.

uptake in cytoplasmic lysosomes, for 90 to 95% of the cell population. In contrast, cells killed by the light-dye combination demonstrated cell death not only by vital stains but also by morphological appearance. Cell to cell contact was greatly decreased, cells were crenated, and membranes appeared pale and thin.

Minimum Photoradiation Time. A series of tubes, each containing 5 × 10^5 retinoblastoma cells and hematoporphyrin, 40 μg/ml, with appropriate controls, were exposed to white light, 6.0 microwatts/sq mm, for periods varying from 1 to 60 min. In 3 separate experiments, the mean time required to cause 50% cell death was 3 min and the mean time to produce 100% cell death was 6 min (Chart 2).

Influence of Serum Concentration on the Photodynamic Activity of Hematoporphyrin. Cells from the Y79 line were cultivated in medium containing either 10 or 25% FCS and subjected to the dye-light treatment with controls. The experiment was repeated with 3 different lots of FCS, 2 specimens of freshly drawn rabbit plasma, 1 specimen of fresh rabbit serum, and 1 specimen each of rabbit serum and FCS which were not heat inactivated. When media contained 25% serum or plasma, the cytotoxic effects of hematoporphyrin and light were sharply reduced (Chart 1). The results were not affected by heat inactivation of the sera. This inhibitory action varied for individual animal sera, and in repeated trials the value for 50% cell death fell between 40 and 1280 μg/ml. Furthermore, the slope of the curve was decidedly less than that of the standard, which always rose asymptotically to 100% cell death, as depicted in Charts 1 and 2. The plateau effect in the graph for 25% FCS is probably not significant, and the S.E., which was greatest (±18%) near 40% cell death, could accommodate a positive slope in this region. Control cultures exposed to light without hematoporphyrin or with hematoporphyrin but kept in the dark showed only a 5 to 10% background cytotoxicity. One of 3 lots of FCS had no inhibitory effect on hematoporphyrin activity.

Sensitization Period for Retinoblastoma and Hematoporphyrin. A suspension of Y79 retinoblastoma cells was sensitized with hematoporphyrin at 80 μg/ml in a series of tubes, and at varying times the cells were centrifuged and washed twice in $RPMI_{10}$. Sensitized cells and controls were then exposed to light as before and tested for viability. The rate of cell death after 1.5 and 3.5 hr cell contact was, respectively, 22 and 99.8%, and it was 100% for periods of 6, 9, and 24 hr cell contact.

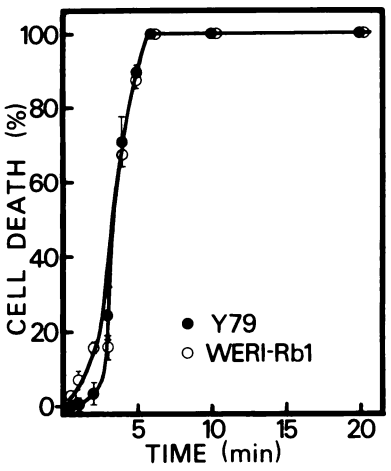

Chart 2. Percentage of cell death (less control value of 5% dead cells initially present) for retinoblastoma cell lines Y79 and WERI-Rb1 sensitized with hematoporphyrin, 40 μg/ml, for 6 hr at 37°. They were exposed to a 15-watt cool white fluorescent light (6.0 microwatts/sq mm), at a distance of 10 cm, for varying times.

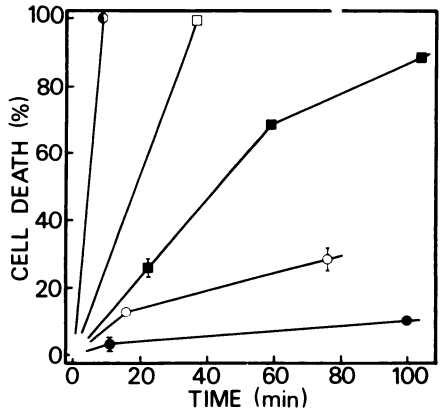

Chart 3. Percentage of cell death of Y79 cells due to sensitization with hematoporphyrin, 40 μg/ml, and different periods of light exposure at 6.0 μwatts of irradiance per sq mm. Immediately after cells were combined with dye, they were exposed to light in separate tubes for 9 (●), 12 (○), 17 (■), 32 (□), or 48 min (◐). Tests for cell viability at different periods (*abscissa*) were made starting from the time that light exposure was terminated. Two of the *graphs*, the 48- and 32-min preparations, are represented by a *single point*; the first determination for these had already reached 99.8 and 99.3% cell deaths, respectively. All determinations are corrected for an initial background dead cell population of 5.6% and, therefore, are shown to originate from 0%. The S.E.'s were calculated from cell counts made in quadruplicate and are shown as *bars* only where they are greater than ±1.5%.

Sensitization Period with Hematoporphyrin without Washing Cells. In all previous experiments, the standard procedure was to incubate retinoblastoma cells for at least 6 hr or overnight, expose them to light (for 30 or 60 min), and then reincubate them overnight before applying the trypan blue dye exclusion test. In this experiment, the cell: dye mixtures in hematoporphyrin, 40 μg/ml, were given varying periods of exposure to light immediately after mixing and were tested for cell survival, with no further incubation period. The results, as given in Chart 3, demonstrate that the sensitization period for obtaining 100% cell death, apart from the time spent during light exposure, can be reduced to less than 10 min if the light exposure is sufficiently long. With exposure of sensitized cells to 6.0 microwatts of irradiance per sq mm, greater than 99% cell death was reached in 9 min, for an exposure period of 48 min, and it was reached in 33 min after a 32-min exposure time.

Preliminary Exposure of Hematoporphyrin to Light. Hematoporphyrin alone, at 80 μg/ml, was exposed to light at 6.0 microwatts/sq mm for 1 to 60 min and 2 to 16 hr. The dye was then used to sensitize Y79 cells for photoradiation (32 min light exposure and overnight incubation). For periods of 8 hr or less of prior light exposure, hematoporphyrin was fully active in its photodynamic action against retinoblastoma, causing 100% cell death; exposure of the dye to light for 16 hr before sensitizing the cells reduced the activity of the dye to 62% cell death.

DISCUSSION

The present study demonstrates that retinoblastoma cells are highly sensitive to the photodynamic action of white light and hematoporphyrin. When sensitization periods of cell-dye contact were 6 hr or longer, the lowest concentration of hematoporphyrin necessary to kill an entire population of 10^6 cells was 1.67×10^{-5} M (10 μg/ml), and the lowest dose of light exposure required was an irradiance of 6.0 microwatts/sq mm for 6 min. Cells, either exposed to light in absence of dye or mixed with the dye without being exposed to light, even at 100 times greater concentrations for a 96-hr period, suffered no damage. It was found that a 6-hr sensitization period was unnecessary if the period of light exposure was adequate. Exposure for 48 min to the standard dose of irradiance was sufficient to kill 99.8% of a cell population in less than 10 min, while 32 min exposure produced 99.3% cell death within 33 min. This time delay between sensitization and photoradiation was quite different if cells were washed free of the dye before exposure to light. With this modification, the cells required at least 3.5 hr of contact with hematoporphyrin if close to 100% cell death of a given population was to be achieved. A shorter period of 1.5 hr cell-dye contact before washing the cells free of dye produced only 22% cell death. Thus, it can be seen that the maximum lethal effect against retinoblastoma cells may be achieved either with the presence of hematoporphyrin in the medium or with sensitized cells that have been washed free of dye but that the former is far more effective.

In addition to adding a new member to the growing list of neoplastic cell lines which are sensitive to the photodynamic action of light and hematoporphyrin derivatives, the present study also demonstrates an exceptionally efficient tool for the study of photosensitive dyes. Since retinoblastoma cells grow in suspension rather than as a monolayer, the loose cell clusters are readily dispersed into small, countable cell groups of less than 10 cells without the use of enzymes or detergents. This permits rapid and accurate quantitative counts of living and dead cells by the trypan blue dye exclusion method. Expecially valuable for this procedure is the Y79 cell line because of its rapid proliferation rate, a cell doubling time of 33 hr (13).

The demonstrated serum- or plasma-inhibitory effect on hematoporphyrin indicates that the dye is not entirely available to cancer cells when injected into a living host. Apart from the fraction which is taken up by normal cells, there is an additional fraction which is removed from the available supply by plasma factors. Although no effort was made to isolate and test the plasma factors directly responsible for this inhibitory property, there is good reason to believe that hemopexin is primarily responsible for this effect. Hemopexin, a β-glycoprotein with a molecular weight of 57,000 for both human and rabbit, is normally present in plasma in the approximate range of 30 to 50 mg/100 ml (21). With a strong porphyrin-biding capacity (14, 16), the main function of hemopexin may be to bind and carry heme to the liver for its ultimate elimination (16). At 25% serum concentration, the inhibition of hematoporphyrin activity was considerable, and it would be expected to be much greater at 100% concentration *in vivo*.

Hematoporphyrin's photosensitive property in biological systems has had a long history as a practical tool for the diagnosis of cancer, but a resurgent interest in this system in recent years has encouraged new research for its possible therapeutic value against cancer (1, 3, 5, 7, 8, 25). Other photosensitive dyes have been tested for this purpose (2, 22), and additional agents will undoubtedly become available with time. Use of retinoblastoma cell lines will prove to be an efficient laboratory model for the study of these agents. At present, hematoporphyrin derivatives provide a rich and valuable source of photosensitive dyes that may be examined for eventual trials in the phototherapy of cancer.

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