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Comparison of Mutagenicity and Induction of Sister Chromatid Exchange in Chinese Hamster Cells Exposed to Hematoporphyrin Derivative Photoradiation, Ionizing Radiation, or Ultraviolet Radiation[1]

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ABSTRACT

Cell culture studies have been performed to compare the mutagenic potential and the induction of sister chromatid exchanges for hematoporphyrin derivative photoradiation, ionizing radiation, and UV radiation. The mutation frequency in Chinese hamster ovary cells at the hypoxanthine-guanine phosphoribosyltransferase locus was measured using resistance to 6-thioguanine. Phenotypic expression time prior to mutation selection was also examined. Treatment with either X-rays or UV was effective in producing mutants resistant to 6-thioguanine, but treatment with hematoporphyrin derivative photoradiation (at comparable toxicity levels) did not induce any mutagenic activity above background levels. The hematoporphyrin derivative incubation and photosensitization conditions used in this study did induce sister chromatid exchanges at frequencies comparable to those induced by X-rays but at lower frequencies than for UV treatments.

INTRODUCTION

The results from initial clinical trials utilizing HPD[3] PRT for the treatment of solid tumors are encouraging (10, 11, 16). The preferential retention of HPD in malignant tissue when compared to surrounding normal tissue (18, 19, 26) and the photodynamically induced generation of cytotoxic oxygen species by HPD when illuminated with visible red light (34) account for the effectiveness of this therapy. Advances in the development of both external and interstitial methods of light delivery (12) allow HPD PRT to be used in the treatment of malignant lesions of the lung, brain, bladder, eye, head and neck, and skin (13). Currently, there are more than 15 centers which are actively undertaking clinical trials using HPD PRT.[4] As a consequence of the increasing clinical use of HPD PRT, there is a significant need to examine and document potential side effects of this therapy.

The cytotoxic response in tissue following HPD PRT is extremely rapid (12, 13). Vascular disruption and tissue necrosis are evident within 24 hr of treatment. These observations suggest that the majority of HPD PRT-induced damage is membrane related. Cell culture studies documented that the interaction of HPD and visible light does induce membrane damage in the form of inhibited transport of nucleosides and amino acids as well as loss of permeability barriers (23). However, while the cellular membrane may be a primary site for HPD PRT-induced toxicity, there are several studies which report that damage to components of the cell nucleus can be induced by HPD photosensitization. Specifically, damage to DNA in the form of alkali-labile lesions (17), single- and double-stranded breaks (15), induction of SCE (8), and chromosome aberrations (14) can be observed following HPD photosensitization. The documentation of DNA damage had led to inquiries regarding the mutagenic and carcinogenic potential of HPD PRT (15).

In the present study, we have compared cytotoxicity, mutagenic potential (resistance to 6-TG), and induction of SCEs in CHO cells exposed to either HPD photosensitization, ionizing radiation, or UV radiation.

MATERIALS AND METHODS

Drugs. HPD was obtained from Oncology Research and Development, Inc., Cheektowaga, N. Y., as a sterile solution dissolved in 0.9% NaCl solution at a concentration of 5 mg/ml. The purine analogue 6-TG was obtained from Sigma Chemical Co., St. Louis, Mo.

Light and X-Ray Sources. A parallel series of soft white 30-watt fluorescent bulbs (Sylvania, F30T12), enclosed on top with a sheet of clear Plexiglas and filtered with a Mylar film (Rubylith SR-3; Ulano Corp., Brooklyn, N. Y.), was used as the light source for all HPD photosensitization experiments. A treatment stand (consisting of one clear and one frosted sheet of Plexiglas) was placed 5 cm above the light source. Dishes were placed on top of the treatment stand, and cells were illuminated from below. The emission spectrum of this light source was examined using a scanning monochromator (American ISA, Metuchen, N. J.) and was determined to have a range of 570 to 650 nm, with a peak output at 620 nm. The light intensity at the treatment site was measured with a radiometer-photometer (EG & G, Inc., Electro-Optics Division, Salem, Mass.) and was determined to be 0.35 milliwatt/sq cm, which corresponded to a dose rate of 210 J/sq m/min.

A 30-watt germicidal lamp (Sylvania, G30T8) was used for UV irradiation. This lamp emits primarily at 254 nm (32). Dishes were placed 45 cm below the light source, and the dish covers were removed prior to treatment. The light intensity at the treatment site was measured with a Blak-Ray ultraviolet photometer (Ultra-Violet Products, Inc., San Gabriel, Calif.) and was determined to be 0.2 milliwatt/sq cm, which corresponds to a dose rate of 120 J/sq m/min.

X-rays were obtained using a General Electric Maxitron X-ray unit. The X-rays were produced at 300 kVp and 20 ma and were filtered with 2-cm copper. The dosimetry was measured with a Condenser R-Meter (Victoreen, Inc., Cleveland, Ohio), and the dose rate at the treatment site was determined to be 155 rads/min.

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[3] The abbreviations used are: HPD, hematoporphyrin derivative; PRT, photoradiation therapy; SCE, sister chromatid exchange; 6-TG, 6-thioguanine (2-amino-6-mercaptopurine); HGPRT, hypoxanthine-guanine phosphoribosyltransferase.

[4] T. J. Dougherty, personal communication.

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Cell Line, Cell Cultivation, and Survival Assays. CHO cells were used for all experiments in this study (17, 20). The cells were maintained as a suspension culture in Ham's F-10 medium (Irvine Scientific, Irvine, Calif.) supplemented with 10% heat-inactivated FCS and antibiotics (penicillin and streptomycin). The procedure used for the treatment of cells and the subsequent determination of survival by colony formation have been described previously (20). Appropriate numbers of cells were plated onto 60-mm plastic Petri dishes so that between 50 and 250 macrocolonies would be formed following each experimental procedure. Following a 4-hr incubation at 37° to allow for cellular attachment, the medium in the dishes was removed, and the cells were rinsed once with F-10 medium lacking serum. In the case of HPD PRT experiments, the cells were then incubated at 37° for either 1 or 12 hr in F-10 medium supplemented with 1, 5, or 10% FCS and containing HPD at a concentration of 25 or 50 μg/ml. Following incubation, the HPD-containing medium was removed, and the cells were rinsed once in serum-free medium. The cells were exposed to red light at room temperature and were then refed with fresh F-10 medium containing 10% FCS. The dishes were incubated at 37° for 7 to 9 days, and the survival levels were determined from colony formation. In experiments involving X-rays or UV radiation, the cells were treated in monolayer after being rinsed once with F-10 medium lacking serum. Following treatment, the cells were refed with fresh F-10 medium containing 10% FCS and returned to a 37° incubation for colony formation. Three dishes were treated at each dose point in every experiment.

Mutation Assays. The procedure which was used to measure mutation induction at the HGPRT locus has been described previously (2, 29). Briefly, 5×10^5 CHO cells were plated onto 60-mm plastic Petri dishes and then incubated at 37° for 4 hr to allow for cell attachment. The cells were then exposed to either HPD photoradiation, X-irradiation, or UV irradiation as described above. Immediately following treatment, the cells were refed with hypoxanthine-free F-10 medium containing 10% dialyzed FCS. The cells were kept in exponential growth by subculturing every 2 days. After an expression period of 9 days, the cells were replated in 100-mm dishes (2×10^5 cells/dish) containing hypoxanthine-free F-10 medium supplemented with 10% dialyzed FCS and 10 μM 6-TG. Ten dishes at each dose point were examined in each experiment. These dishes were incubated at 37° for 8 days, and the resultant colonies were fixed and stained. The plating efficiency for each dose point was determined at the time of selection for 6-TG resistance. All mutation frequencies have been corrected for plating efficiency.

For experiments designed to examine the effect of phenotypic expression time, the cells were treated to approximately a 15 to 25% survival level. The mutation assay was identical to that described above except that treated cells were plated into selection medium containing 10 μM 6-TG at 48-hr intervals following treatment.

SCE. The method used to assay for SCEs has been reported previously (1). Briefly, 5 to 10×10^5 cells were exposed to HPD photoradiation, X-rays, or UV as described above. Immediately following treatment, the cells were refed with F-10 medium containing 10% FCS and supplemented with 5-bromo-2-deoxyuridine (3 μg/ml). The cells were then incubated at 37° in the dark for 27 or 36 hr. Colcemid (0.06 μg/ml) was added to each dish for the final 3 hr of incubation. Chromosome preparations were obtained, and then slides were stained for 15 min with Hoechst Dye No. 33258 (0.5 μg/ml). After the slides were exposed for 5 min to light from a 500-watt mercury arc lamp, they were rinsed with distilled water and then stained with 3% Giemsa. The number of SCEs per chromosome was determined on the basis of at least 25 intact and differentially stained metaphases per experiment. A 2-tailed *t* test was used for statistical analysis (24), and the frequency of first, second, and third mitoses was determined by sister chromatid differentiation (33).

RESULTS

Survival Measurements. The survival curves obtained for exponentially growing CHO cells exposed to either HPD photoradiation, X-rays, or UV are shown in Charts 1 and 2. The treatment conditions used in this study induced up to 95% cytotoxicity when assayed using standard colony formation techniques. All curves were fitted by eye and illustrated a characteristic shoulder region at low doses followed by exponential killing at higher doses. The plating efficiency ranged from 70 to 90% throughout the study. There was no detectable cytotoxicity for cells incubated with HPD for up to 12 hr (in the absence of light) or for cells exposed only to red light. Lower fluences of light were required to induce comparable levels of phototoxicity when HPD incubation concentrations and/or HPD incubation times were increased. Conversely, higher fluences of light were required to induce comparable levels of phototoxicity when the serum concentrations in the incubation medium were increased.

Mutation Measurements. Chart 3 shows the mutation frequency (measured by resistance to 6-TG) as a function of survival for treated cells. An expression period of 9 days was used when assaying for mutation frequencies following treatment. Treatment with either X-rays or UV inducted a dose-related increase in mutation frequency. The background level for spontaneous

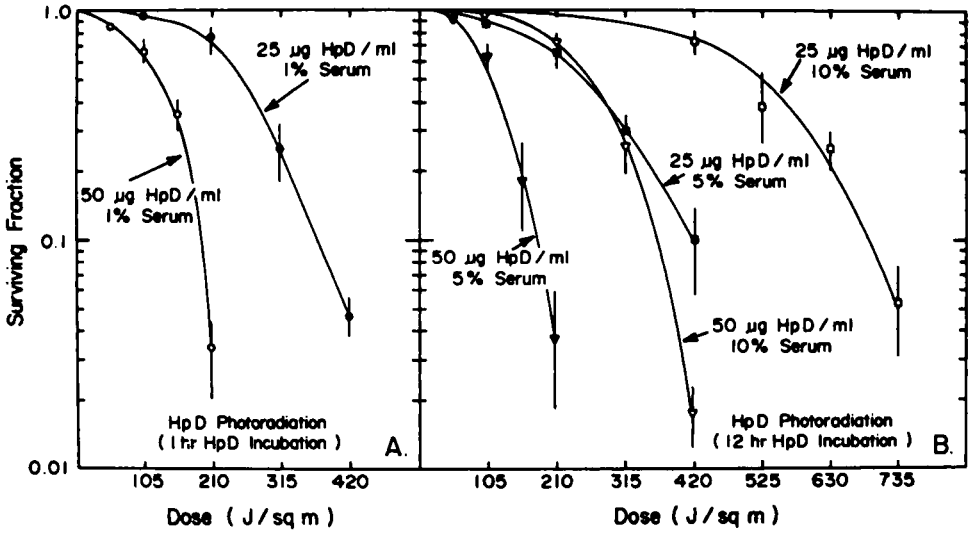

Chart 1. Surviving fraction of CHO cells as a function of treatment dose. Cells were exposed to photoradiation following either a 1-hr (*A*) or 12-hr (*B*) HPD incubation. The HPD concentration during incubation was either 25 or 50 μg/ml, and the percentage of serum in the incubation medium was either 1, 5, or 10%. Each *point* represents the mean for 3 to 5 experiments. Plating efficiency, 70 to 90%; *bars*, S.E.

mutations was determined to be 5 to 20 per 10^6 survivors. There was no observed mutagenic effect for CHO cells exposed to the HPD photoradiation at any of the drug levels or incubation conditions examined. Likewise, exposure of cells to HPD alone or to red light (2.1×10^3 J/sq m) alone was also nonmutagenic.

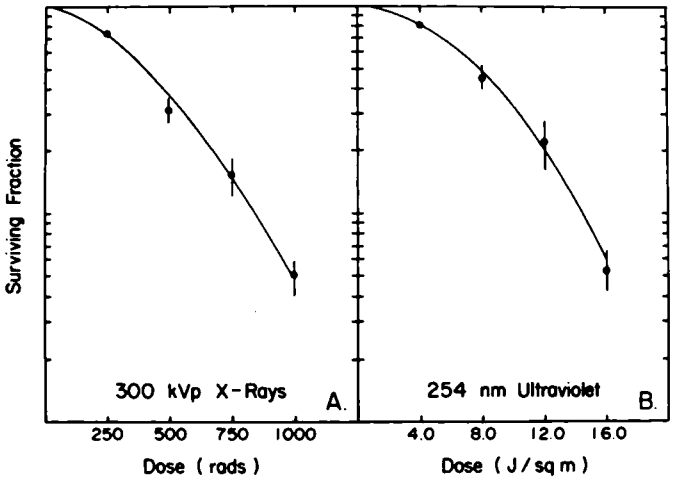

Chart 2. Surviving fraction of CHO cells as a function of treatment dose. Cells were exposed to either ionizing radiation (*A*) or UV radiation (*B*). Each *point* represents the mean for 3 experiments. Plating efficiency, 70 to 90%; *bars*, S.E.

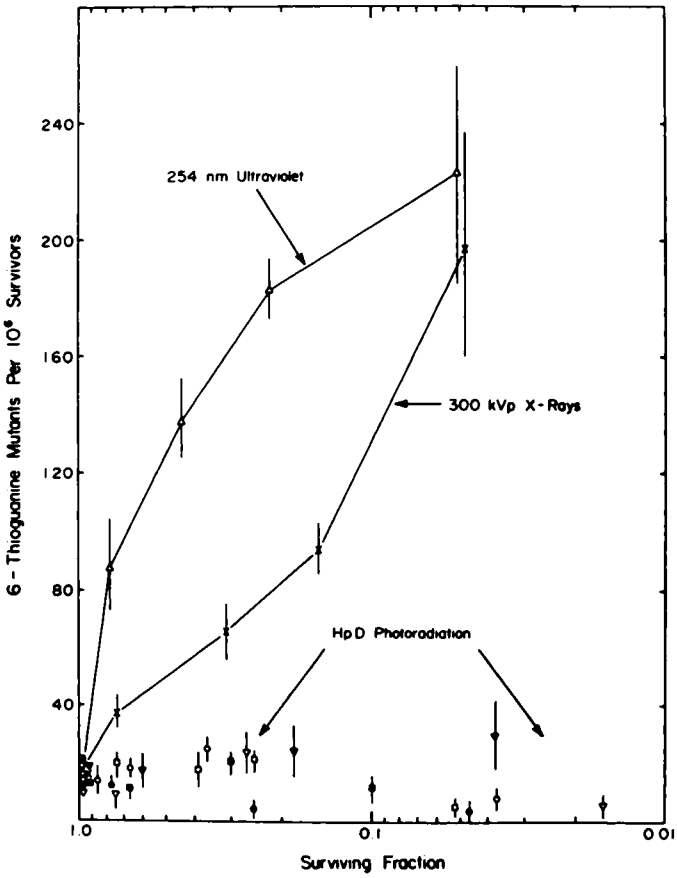

Chart 3. Mutation frequency (6-TG-resistant mutants per 10^6 survivors) measured as a function of surviving fraction of CHO cells treated with either UV (△), X-rays (×), or HPD photoradiation (○, ●, ▽, ▼, □, and ■). *Symbols* used to denote HPD photoradiation are identical to those used in Chart 1. Each *point* represents the mean for 3 to 5 experiments; *bars*, S.E.

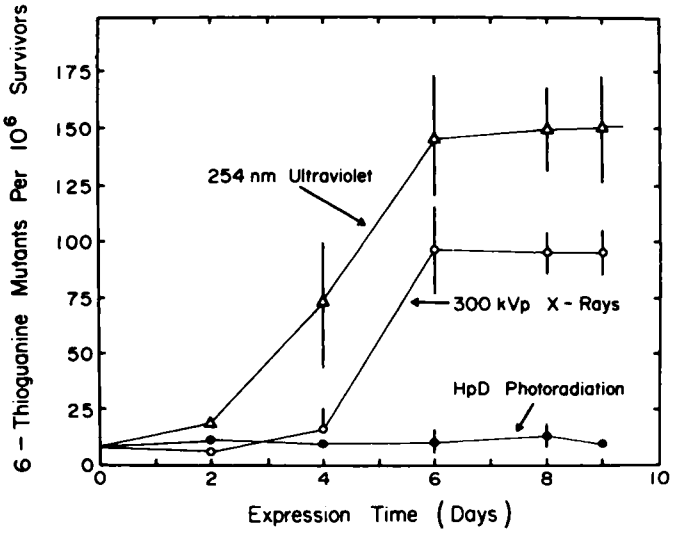

Chart 4. Mutation frequency (6-TG-resistant mutants per 10^6 survivors) measured as a function of phenotypic expression time (days) following treatment of CHO cells with either UV (△), X-rays (○), or HPD photoradiation (●). Cells were treated at a dose which induced 75 to 85% cytotoxicity. Each *point* represents the mean for 2 to 3 experiments; *bars*, S.E.

Chart 4 shows the mutation frequency as a function of phenotypic expression time. The optimal expression time for CHO cells treated with either X-rays or UV was approximately 6 days. Varying the expression time from 2 to 9 days did not produce any detectable mutations (above background) for cells exposed to HPD photoradiation.

SCE Measurements. Table 1 lists the frequencies of SCEs obtained for all treatment procedures. A minimum of 25 cells were scored at each dose in each experiment. The background frequency ranged from 8 to 10 SCEs/cell, and the SCE frequencies for cells incubated with HPD (in the absence of light) and for cells exposed to red light alone were not statistically different from control levels. A maximal 2-fold increase in SCE frequency was observed for cells exposed to either X-rays or HPD photoradiation. Treatment with UV irradiation was the most efficient at producing SCEs and a 4- to 5-fold increase was observed following 8 J/sq m. The frequency of SCEs for cells exposed to UV doses greater than 8 J/sq m was not documented due to the lack of a representative population of M_2 metaphases caused by a prolonged division delay. This observation has also been observed when scoring SCEs in *Xenopus laevis* fibroblasts exposed to UV (33).

DISCUSSION

The mutagenic frequency of CHO cells exposed to either HPD photoradiation, ionizing radiation, or UV radiation was measured using the HGPRT system (29). This procedure quantitatively assays for mutation at the HGPRT locus on the X-chromosome by culturing for resistance to 6-TG. Both point- and deletion mutations at the HGPRT locus will result in cellular resistance to 6-TG (32). In the present study, 6 *in vitro* HPD photoradiation protocols (combining 2 HPD concentrations, 2 HPD incubation times, and 3 incubation serum concentrations) were examined. There was no increase in mutation frequency (above background levels) for cells exposed to HPD photoradiation at doses which induced up to 95% cytotoxicity. However, both ionizing and UV

Table 1

SCE in CHO cells exposed to HPD photoradiation, ionizing radiation, or UV radiation

Treatment	Dose	No. of cells analyzed	5-Bromo-2-deoxyuridine labeling time (hr)	SCE/cell	5-Bromo-2-deoxyuridine labeling index			p^a
					M_1	M_2	M_3	
Control	0 J/sq m	100	27	8.62 ± 0.39[b]	3	97	1	
	0 J/sq m	50	36	9.76 ± 0.35	0	94	6	
	210 J/sq m	25	36	10.00 ± 0.73	5	95		>0.2
	410 J/sq m	25	36	9.68 ± 0.64	5	95		>0.2
HPD	0 J/sq m	50	27	7.82 ± 0.50	5	95		>0.2[c]
25 μg/ml, 1 hr, 1% serum	0 J/sq m	50	36	8.70 ± 0.44	2	95	3	>0.2[d]
	105 J/sq m	50	27	11.41 ± 0.55	11	74	15	<0.01
	210 J/sq m	50	27	12.88 ± 0.60	17	83		<0.01
	210 J/sq m	50	36	14.22 ± 0.89	4	84	12	<0.01
	315 J/sq m	50	27	12.49 ± 0.55	35	65		<0.01
	315 J/sq m	50	36	12.84 ± 0.71	20	80		<0.01
	420 J/sq m	50	27	12.46 ± 0.54	40	60		<0.01
	420 J/sq m	50	36	11.22 ± 0.55	48	52		<0.05
HPD	0 J/sq m	50	36	9.92 ± 0.31	4	96		>0.2
50 μg/ml, 1 hr, 1% serum	52.5 J/sq m	50	36	11.26 ± 0.51	20	80		<0.05
	105 J/sq m	50	36	16.50 ± 0.93	60	40		<0.01
	157.5 J/sq m	50	36	15.05 ± 0.68	58	42		<0.01
	210 J/sq m	50	36	14.20 ± 0.81	68	32		<0.01
HPD	0 J/sq m	50	36	8.76 ± 0.40	2	94	4	>0.2
25 μg/ml, 12 hr, 10% serum	420 J/sq m	50	36	12.36 ± 0.62	12	84	4	<0.01
	525 J/sq m	50	36	14.08 ± 0.64	13	87		<0.01
	630 J/sq m	25	36	11.32 ± 0.57	60	40		<0.05
	735 J/sq m	25	36	12.90 ± 0.68	60	40		<0.01
HPD	0 J/sq m	50	36	9.22 ± 0.47	8	92		>0.2
25 μg/ml, 12 hr, 5% serum	105 J/sq m	50	36	13.58 ± 0.69	8	90	2	<0.01
	210 J/sq m	50	36	15.06 ± 0.64	15	85		<0.01
	315 J/sq m	50	36	14.02 ± 0.83	60	40		<0.01
	420J/sq m	50	36	14.82 ± 0.77	59	41		<0.01
HPD	0 J/sq m	50	36	10.50 ± 0.45	1	90	9	>0.2
50 μg/ml, 12 hr, 10% serum	105 J/sq m	50	36	11.50 ± 0.62	16	82	2	<0.05
	210 J/sq m	50	36	17.70 ± 0.71	58	42		<0.01
	315 J/sq m	50	36	13.30 ± 0.72	66	34		<0.01
	420 J/sq m	50	36	14.30 ± 0.82	74	26		<0.01
HPD	0 J/sq m	50	36	9.98 ± 0.44	7	84	9	>0.2
50 μg/ml, 12 hr, 5% serum	52.5 J/sq m	50	36	11.12 ± 0.48	20	79	1	>0.2
	105 J/sq m	50	36	18.06 ± 0.98	30	70		<0.01
	157.5 J/sq m	50	36	16.88 ± 0.77	46	54		<0.01
Ionizing radiation	250 Rads	25	27	10.90 ± 0.83	13	85	2	<0.01
	500 Rads	25	27	14.60 ± 0.79	12	83	3	<0.01
	750 Rads	25	27	16.48 ± 1.24	17	83		<0.01
	1000 Rads	25	27	17.56 ± 1.28	40	60		<0.01
UV radiation	4 J/sq m	25	27	21.08 ± 1.93	2	98		<0.01
	4 J/sq m	50	36	35.00 ± 1.69	5	95		<0.01
	8 J/sq m	25	27	38.76 ± 2.98	37	63		<0.01
	8 J/sq m	50	36	52.30 ± 2.60	42	58		<0.01
	12 J/sq m	25	27	ND[e]	>95			
	12 J/sq m	50	36	ND	>95			

[a] Two-tailed *t* test.
[b] Mean ± S.E.
[c] SCE samples analyzed after a 27-hr 5-bromo-2-deoxyuridine incubation were compared to the control determined with a 27-hr 5-bromo-2-deoxyuridine incubation.
[d] SCE samples analyzed after a 36-hr 5-bromo-2-deoxyuridine incubation were compared to the control determined with a 36-hr 5-bromo-2-deoxyuridine incubation.
[e] ND, not determined (no representative M_2 population).

radiation were effective in the production of cells resistant to 6-TG at comparable cytotoxic doses. The mutagenic results for X-ray and UV light were in agreement with studies reported previously (5, 30). A phenotypic expression period of 6 days was determined to be optimal for cells exposed to X-rays or UV light, but there was no observation of mutation induction following HPD photosensitization for phenotypic expression periods ranging from 2 to 9 days posttreatment.

While the HPD photoradiation protocols utilized in the present study were not found to be mutagenic, they did induce up to a 2-fold increase in SCE frequency. The increase in SCEs following HPD photoradiation was of the same magnitude as that observed for cells exposed to ionizing radiation but 2 to 3 times less than that observed for cells exposed to UV radiation. The observation that HPD photoradiation induces SCE formation is in agreement with a previous study which demonstrated an increase in SCE formation following HPD photosensitization of human NHIK 3025 cells (8). The results for X-rays and UV agree with previous

reports that indicate that UV produces a sizeable increase in SCE frequency, while X-irradiation causes only a slight increase in frequency (22). Our results would therefore suggest that, like X-irradiation (24), HPD photoradiation is also a positive but weak inducer of SCEs. The increase in first posttreatment metaphase spreads at increased treatment doses demonstrates that division delay is induced in cells exposed to HPD photoradiation as well as to ionizing or UV radiation. This observation is in agreement with results from a previous study in which a division delay was observed in human NHIK 3025 cells treated with HPD and light (7).

There is generally a positive correlation between SCE induction and mutagenicity, even though the relative efficiency of SCE and mutation induction varies for different treatment modalities (6, 25). However, a recent study which examined mutation induction and SCE frequency in CHO cells exposed to the alkylating agents ethyl methanesulfonate, ethylnitrosourea, methyl methanesulfonate, and methylnitrosourea found that the production of alkylation-induced SCEs did not correlate with mutation induction at the HGPRT locus (28). In our study, no detectable mutagenic induction was observed for HPD-treated cells using conditions which were shown to induce SCEs and which were previously reported to induce single-stranded breaks in DNA (17). Our observations are similar to results obtained for mammalian cells treated with hydrogen peroxide (3, 4). Exposure of cells to hydrogen peroxide is cytotoxic and produces both DNA damage and SCEs, but the treatment is not mutagenic when assayed using the HGPRT system.

Singlet oxygen is reported to be responsible for the cytotoxicity induced by HPD photosensitization (34), and this agent is known to be mutagenic (9). However, as stated above, HPD photosensitization of CHO cells was not determined to be mutagenic. It is possible that the level of nuclear damage and the resulting mutagenic induction frequency following HPD photosensitization was disproportionately low relative to the level of lethal damage induced outside the nucleus. The cell membrane and/or cytoplasm are thought to be major targets of HPD-induced cell killing (13, 23). Localization of HPD is also reported to be associated with the cell membrane and cytoplasm (14, 27). The loss of cell viability observed by the photosensitization of a series of porphyrins (including HPD) is reported to be correlated with damage to membrane transport and loss of normal permeability barriers (23). At the clinical level, HPD PRT induces rapid tissue necrosis, which also implies membrane-related damage (13).

Finally, the lack of detectable mutagenic activity by HPD photosensitization suggests that there will be a relatively low risk of carcinogenic side effects from HPD PRT. However, the photodynamic action of other sensitizers can induce tumors in experimental animals (21, 31) and, therefore, additional studies regarding the potential side effects of HPD PRT are needed. Experiments designed to examine the cellular transformation potential of HPD photosensitization are in progress. *In vivo* carcinogenic studies will have to be performed to determine the long-term effects of HPD PRT.

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