Dye-sensitized Photoinactivation of Tumor Cells *in Vitro*

JUDITH S. BELLIN,* STEVEN C. MOHOS, AND GERALD OSTER

(*Department of Pathology, State University of New York, Downstate Medical Center, and Department of Chemistry, Polytechnic Institute of Brooklyn, Brooklyn, N.Y.*)

SUMMARY

The *in vitro* photodynamic inactivation of a wide variety of tumor cells was studied with various dyes used as sensitizers. The capacity of dyes to act as photosensitizers in this system parallels their capacity to sensitize the photoinactivation of many other substrates and correlates with their capacity to undergo photoreduction. Some dyes are able to inactivate tumor cells in the absence of light. Photodynamic inactivation of the tumor cells destroys their tumor-producing capacity but does not change their dye-binding capacity.

It has long been known that some microorganisms, when stained with certain dyes, can be inactivated with visible light. This process, commonly referred to as photodynamic action, involves the participation of oxygen and consists of a dye-sensitized photoautoxidation of some essential elements of the substrate involved. Many diverse substrates are affected by photodynamic action. These include enzymes (23), transforming principle (1), viruses (11), bacteria (10), and erythrocytes (8). The autoxidation of many simple organic substrates is enormously accelerated by light in the presence of certain sensitizing dyes. A mechanism for the photosensitized autoxidation of *para*-toluenediamine and criteria for the capacity of dyes to act as sensitizers in such a system were established (18). It was found that those dyes which can photosensitize the autoxidation of aromatic amines can also act as sensitizers for the photodynamic inactivation of transforming principle (1).

There is an analogous relationship between photodynamic action and the effect of x-rays in that both phenomena involve autoxidations. For example, ferrous sulfate is autoxidized by the action of x-rays as well as by dyes and visible light; viruses can be inactivated by the action of both agents when oxygen is present. Since some tumor cells are susceptible to the action of ionizing radiation when oxygen is present, they might likewise be inactivated by photodynamic action.

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Menke (16) has shown that both normal and neoplastic cells grown in tissue culture die when subjected to the action of visible light in the presence of a xanthene dye. The criterion of death in this author's publication was a subjective one, and, since relevant controls were omitted, his results could be interpreted as being due to indirect effects, resulting from the photodynamic destruction of nutrients in the culture medium.

The present paper is concerned with the *in vitro* photodynamic inactivation of tumor cells as judged by their inability to elicit tumor growth. In conformity with previous results on the photodynamic inactivation of transforming principle and *para*-toluenediamine, we have found that only certain classes of dyes can act as photosensitizers in this system.

MATERIALS AND METHODS

TUMORS AND DYES EMPLOYED

The tumors used were: the Gardner lymphoma (6C3HED) grown in 20-gram C3H/Hej mice (both the tumor and mice were obtained from the Jackson Memorial Laboratory); Sarcoma 180 and Ehrlich ascites tumor, both obtained from Dr. H. T. Sugiura at the Sloan-Kettering Institute and both grown in 20-gram Swiss mice from the pathogen-free colony at the State University of New York Downstate Medical Center; mammary adenocarcinoma, dbrB, grown in DBA/1 mice, both obtained from the Jackson Laboratory.

Dyes (histological grade) were obtained from Eastman Kodak Company. The dye concentrations employed were of the order of 10^{-5} M, so that

the small amounts of added salts which these commercial samples contained should not influence the results obtained.

Procedures for Photodynamic Inactivation

In the case of solid mouse tumors (6C3HED and dbrB).—Growths were removed with aseptic precautions, and a suspension of tumor cells was prepared by pressing the tumor through a 40-mesh Monel metal sieve into physiological saline adjusted to pH 7.4. The suspension was diluted with the same medium to give suspensions containing 10^6 cells per ml. as determined by hemocytometer counts. All cell suspensions were routinely tested for bacterial contamination by culture in trypticase soy broth at 37° C. Aliquots of the counted suspension were pipetted into four sterile test tubes, to two of which were added aliquots of a 10^{-3} M sterile stock solution in saline of the dye under study. The final dye concentration was 10^{-5} M, except in those experiments in which it was desired to investigate the effect of various dye concentrations. A dye concentration of 10^{-5} M was routinely employed, because this concentration gives an optical density of about one for most dyes at their respective absorption maxima. Unit optical density gives nearly optimal photometric efficiency. Of the resulting cell suspensions two (with and without dye) were kept in the dark at room temperature as controls, and two with and without dye were illuminated in test tubes at a distance of 15 cm. from the front surface of a 500-watt TDC brand slide projector.[1] This projector has a built-in heat filter and was equipped with a Corning Number 3070 filter to eliminate radiation below 400 mμ. There was no detectable rise in temperature of the illuminated samples. The samples were illuminated for 30 minutes, except where otherwise noted.

The suspensions were then centrifuged in a size 1, model CM International Centrifuge, with a #240 head, at 800 r.p.m. for 10 minutes, after which the sediments were resuspended in their original volume of sterile saline containing 100 units penicillin/ml.

In the case of ascites tumors (Sarcoma 180, Ehrlich ascites).—Tumor cell suspensions were harvested 5 days after inoculation as milky-white suspensions, which were tested as above for bacterial contamination. To diluted suspensions, containing 10^6 cells per ml. of pH 7.4 saline, dye was added to a concentration of 10^{-5} M, and aliquots were illuminated, centrifuged, and resuspended in saline containing 100 units penicillin/ml, as detailed above.

[1] Manufactured by Bell and Howell Company, Chicago, Illinois.

Determination of Cell Viability: Tumor-producing Capacity

The samples of 6C3HED and dbrB tumor cells were tested for their tumor-producing capacity by subcutaneous injection, 0.5 cc. of each suspension being injected into each of four mice in the right or left axilla or groin. The animals were examined daily for signs of tumor growth, and the earliest day such growth became palpable was noted. The increase in tumor size as measured by palpation was charted until the mice died. On autopsy tumor-bearing mice showed huge localized tumor masses at the sites of injection. Previous experiments in our laboratory have shown by methods of serial dilution that, under the experimental conditions outlined above, Gardner lymphoma cannot be successfully transplanted if fewer than 100 viable cells are injected. Since we implanted 500,000 cells, the fact that in "inactivated" samples no tumor growth was noted in more than 30 days means that in such samples fewer than 0.02 per cent of the cells could have survived photodynamic action. The suspensions of Sarcoma 180 and of Ehrlich ascites tumor cells were tested by the intraperitoneal injection of 0.5 cc. (500,000 cells) into each of four mice. The first signs of tumor growth, determined by the appearance of ascites, were usually noted in 4–5 days, and death followed about 10 days later.

Binding of Dyes to Tumor Cells

Fresh suspensions of 6C3HED cells, obtained as noted above, were washed with pH 7.4 saline and resuspended to a concentration of 10^7 cells/ml of suspension; 0.02 ml. of 10^{-3} M dyes were added to duplicate 2-ml. aliquots of this suspension. One sample was illuminated for 30 minutes, and one was kept as a dark control at room temperature. Two other samples of the suspension, to which no dye had been added, served as illuminated and dark controls. After illumination all samples were centrifuged at 800 r.p.m. for 10 minutes; the resulting supernatants (which were not turbid) are referred to as S1. The sedimented cells were washed with pH 7.4 saline, and the resulting supernatant was labeled S2. By means of a Zeiss spectrophotometer, the optical density at the absorption maximum of each dye in question was then determined in (*a*) the solution containing only 10^{-5} M dye in saline, (*b*) supernatant S1, and (*c*) supernatant S2. From the difference in optical density of the appropriate solutions and the molar

extinction coefficient of the dye in question, the amount of dye bound to the cells was determined.

RESULTS

Photodynamic inactivation of tumor cells.—In Table 1 are summarized experiments demonstrating the photodynamic effect on 6C3HED tumor cells of thirteen dyes which were chosen as representative members of the various families of dyes; all were used in 10^{-5} M concentration. The values listed represent averages for eight to twelve determinations. The standard error of these determinations varied from zero to ± 0.4. The first six dyes listed were able to photosensitize the inactivation of tumor cells. It is evident that the participation of both dye and light is required for the process of inactivation to occur: neither light itself, nor the presence of dye *per se*, was able to reduce the tumor-producing capacity of the cells. In another experiment (data not illustrated) it was shown that irradiation of a solution of proflavine in saline did not subsequently inactivate the cells. Tumor cells which have been photoinactivated differ from viable cells in much the same way as do cells which have been subjected to x-ray irradiation (15): frequently the nuclei appear shrunken and the cytoplasm swollen, with a considerable amount of "blebbing" of cytoplasmic material. The next five dyes listed in Table 1 inactivated the tumor cells even in the absence of light ("dark effect"), thus precluding the observation of a possible photodynamic action; methyl green produced some retardation in the absence of light; and congo red was totally without effect. The observed slight retardation in tumor growth produced in the absence of light by eosin Y, thionine, and neutral red could not be quantitatively related to the percentage of tumor cells killed with our methods.

In Table 2 are listed analogous experiments with Sarcoma 180, Ehrlich ascites, and dbrB tumor cells. It can be seen that the dyes tested exhibited a photodynamic effect on this wide variety of tumor cells just as they do in the case of Gardner lymphoma: the photodynamic action, therefore, is not confined to specific tumor cells. In contrast to observations on Gardner lymphoma cells (cf. Table 1), rose bengal did not affect Ehrlich ascites cells or Sarcoma 180 cells in the absence of light; proflavine had some "dark action" on Ehrlich ascites cells.

In a series of experiments with methylene blue, proflavine, and rose bengal used as sensitizers, it was established that, in suspensions containing 10^6 cell per ml. and 10^{-5} M dye, all the tumor cells were inactivated in 10 minutes of exposure. These experiments are summarized in Table 3. With 10^{-5} M methylene blue or proflavine, 5 minutes' illumination gave only partial inhibition (100 per cent takes, but after an increased latent period); with 10 minutes of illumination at the same dye concentration *complete* tumor inhibition was observed. Rose bengal, as previously noted, had a "dark effect," and was able to inactivate the tumor cells even in the absence of light. Methylene blue had a similar, but less pronounced effect: if, instead of being washed after exposure, in order that they be free of excess dye, the cells were injected in a suspension containing 10^{-5} M methylene blue, tumor inhibition was noted.

TABLE 1

PHOTODYNAMIC INACTIVATION OF TUMOR CELLS *in Vitro*

(Gardner Lymphosarcoma)

Dye	Tumor viability (Number of days before tumor becomes palpable)			
	Without dye		With dye	
	No light	Light	No light	Light
Eosin Y	11	12	15	>30
Methylene blue	10	11	11	>30
Thionine	11	12	13	>30
Proflavine	9	10	10	>30
Acridine orange	11	12	12	>30
Neutral red	11	12	14	>30
Rose bengal	11	11	>30	>30
Crystal violet	11	12	>30	>30
Thioflavine TG	11	12	>30	>30
Janus green B	11	11	>30	>30
2,6-dichloroindophenol	11	12	>30	>30
Methyl green	11	12	18	>30
Congo red	11	11	12	12

Dyes used in 10^{-5} M concentration; 500,000 tumor cells implanted.

To test whether the presence of oxygen is necessary for the photodynamic inactivation of the tumor cells, attempts were made to eliminate oxygen from the solution. Bubbling with nitrogen denatures the cells, as shown by their inability to cause tumor growth on transplantation. Therefore, lymphoma cell suspensions in a Warburg apparatus were flushed across the surface of the suspension with oxygen-free nitrogen for 45 minutes prior to and during 15 minutes of subsequent illumination. A photodynamic effect was still observed, and control suspensions did not show any reduced viability. This was presumably due to the fact that there was still enough oxygen

available to permit photodynamic action to occur (a concentration of only 5×10^{-5} M oxygen is sufficient to allow for photodynamic action [18]).

The capacity of the Gardner lymphoma cells to bind dyes was investigated as detailed above. In the case of eosin Y, for instance, the optical density of a 10^{-5} M solution was 0.840 at 516 mμ. After 30 minutes' equilibration in the dark the supernatant solution (S1) had an optical density of 0.622, from which we subtract 0.044, the optical density of the supernatant of a cell suspension equilibrated with saline in the absence of dye. The optical density of the dye in the supernatant, therefore, was 0.578, representing a concentration of 6.88×10^{-6} M dye. The amount of dye absorbed by the cells was 3.12×10^{-9} moles/ml (31.2 per

TABLE 2

PHOTODYNAMIC INACTIVATION OF TUMOR CELLS *in Vitro*

(Other Tumor Types)

TUMOR	DYE	TUMOR VIABILITY (Av. survival of eight mice after tumor implant in days)			
		Without dye		With dye	
		No light	Light	No light	Light
Sarcoma 180 (500,000 cells, I.P.)	Thionine	15	14	14	>67
	Rose bengal	15	14	15	>67
	Proflavine	14	14	14	>67
Ehrlich ascites (500,000 cells, I.P.)	Thionine	14	13	15	>35
	Methylene blue	14	13	12	>35
	Rose bengal	14	13	14	>35
	Proflavine	13	13	18	>35
		Number of days until tumor palpable			
Mammary adenocarcinoma (dbrB) (2.6×10^6 cells s.c.)	Thionine	11	10	14	>67
	Eosin Y	11	10	11	>67

Dyes used in 10^{-5} M concentration.

TABLE 3

PHOTODYNAMIC INACTIVATION OF 6C3HED CELLS *in Vitro*: EFFECT OF VARYING EXPOSURE TIME AND DYE CONCENTRATION

DYE	CONCENTRATION (M)	EXPOSURE TO LIGHT (MIN.)					
		0	5	10	20	30	45
		Tumor viability (days until tumor palpable)					
None	—	9	—	—	—	—	10
Methylene blue	10^{-5}	9	14	>30	>30	>30	>30
"	2.5×10^{-5}	10	—	>30	—	—	—
"	5×10^{-5}	14	—	>30	—	—	—
Proflavine	10^{-5}	10	13	>30	>30	>30	>30
"	2.5×10^{-5}	13	—	>30	—	—	—
"	5×10^{-5}	10	—	>30	—	—	—
Rose bengal	10^{-5}	>30	>30	>30	>30	—	—

500,000 tumor cells implanted; dashes indicate not tested.

cent of the dye in solution), or, since the suspension contained 13.3×10^6 cells/ml, 2.34×10^{-16} moles were absorbed per cell. The results are summarized in Table 4. "Vital" dyes, such as acridine orange, did not stain light-inactivated cells to any greater extent than they did those cells which had been kept in the dark. Congo red (which in no way affects the tumor-producing capacity of the cells) was able to stain the tumor cells at least as well as did the photosensitizing dyes.

TABLE 4

BINDING OF DYES TO 6C3HED TUMOR CELLS

Dye	Dye bound (Moles dye bound/cell) $\times 10^{16}$		Dye remaining in cells after one wash (Moles dye bound/cell) $\times 10^{16}$		Dye extracted in one wash (% of dye originally bound)		Photodynamic action (+) or dark action (D)	Oxidation reduction potential
	Dark	Light Exposed	Dark	Light Exposed	Dark	Light Exposed		
Eosin Y	2.34	2.75	1.24	1.78	47.0	35.3	+	
Methylene blue	5.66	3.67	5.32	2.19	6.0	40.4	+	−.005
Thionine	3.29	4.60	1.75	2.85	46.8	38.1	+	+.063
Proflavine	4.62	4.42	3.55	3.96	23.2	10.4	+	−.5
Acridine orange	4.93	5.41	3.77	4.18	23.5	23.7	+	
Neutral red	5.18	6.17	5.55	4.82	4.4	21.8	+	−.320
Rose bengal	7.04	6.03	6.36	6.02	10.2	0	D	−.15
Crystal violet	5.85	5.88	4.38	4.39	25.1	25.4	D	
Thioflavine TG	2.66	2.43	—	—	—	—	D	
Janus green B	3.78	5.90	2.45	5.60	64.9	5.1	D	−.275
Methyl green	5.76	6.31	5.02	5.79	12.9	8.2	D	
2,6-Dichloroindophenol							D	+.19
Congo red	5.73	6.02	4.62	4.49	19.4	25.4		

DISCUSSION

The capacity of dyes to sensitize the photodynamic inactivation of tumor cells parallels their capacity to sensitize the photodynamic oxidation of organic amines (18) and of transforming principle (1). Thus, eosin Y (a xanthene dye), methylene blue and thionine (thiazine dyes), proflavine and acridine orange (acridine dyes), and neutral red (an azine dye) are able to inactivate photodynamically *para*-toluenediamine and transforming principle as well as tumor cells. By contrast, congo red (an azo dye) is unable to photosensitize the inactivation of any of these substrates. As pointed out in previous publications (1, 18), the capacity of dyes to sensitize photodynamic inactivation is correlated positively with their capacity to undergo photoreduction and hence to form electronically excited molecules in a long-lived metastable state. On the basis of previous experiments (1, 18) one would expect rose bengal (a xanthene dye), crystal violet (a triphenylmethane dye), and thioflavine TG (a thiazole dye) to act as photosensitizers, whereas janus green B and 2,6-dichloroindophenol would not be expected to sensitize. However, the fact that these particular dyes are able to inactivate tumor cells in the absence of light precludes the observation of a possible photodynamic effect. It is possible that at lower concentrations this dark effect might be abolished. The literature contains many references (5, 12) to the *in vitro* effects of redox indicators such as methylene blue, thionine, and 2,6-dichloroindophenol on the respiration and glycolysis of tumor and other tissues. It was stated (12) that the redox potentials of the dyes are important in this respect. However, our results show no connection between the redox potential of the dyes and their capacity to inactivate tumor cells in the dark.

The *in vivo* tumor-inhibiting effect of various dyes has been investigated by many authors. Thus, Brooks (3) found that, of many dyes tested, only methylene blue showed some tumor-inhibiting effect and that, since the injection of Ringer solution caused almost the same inhibition, this effect was not very convincing. Lewis *et al.* (13, 14) and other authors have reported that, of many acridine compounds tested, acridine orange was the most effective in causing tumor regression. Similarly, Goldie *et al.* (9) have found that the addition of 10^{-3} M acriflavine (of which proflavine is the major constituent) to Sarcoma 180 cells

in vitro prevented their subsequent proliferation. The dye concentrations used in all these studies were many times higher than those employed here. Nevertheless, it is worth noting that no such tumor-inhibiting effect of acridine orange or proflavine was observed by us. The tumor-inhibiting effect of rose bengal which we have noted has been previously reported in the literature (21).

The capacity of the photosensitizing dyes to inactivate tumor cells is a nonspecific phenomenon. Those dyes which act as sensitizers do so on a wide variety of tumor cells (cf. Tables 1, 2). The photodynamic action takes place rapidly, since all the tumor cells in suspension are inactivated in 10 minutes (cf. Table 3). The addition of 10^{-5} M dye (which gives an optical density of 1.36 in the case of methylene blue, and of 0.29 for proflavine at their respective absorption maxima) is thus sufficient to produce a rapid inactivation of the tumor cells. Greater concentration of dye or a more prolonged period of illumination is not necessary.

In a simple chemical system photodynamic action is a photo-oxidation. In such a system containing, for example, an organic amine as a substrate, this can easily be demonstrated, because in such a system the exclusion of oxygen abolishes the photodynamic effect. It can be shown that, since a concentration of only 5×10^{-5} M oxygen is necessary for a maximum rate of photo-oxidation, the dye molecules participating must be long-lived species (18). The addition of a mild reducing agent such as glutathione or cysteine temporarily abolishes photo-oxidation of the amine, because photo-oxidation of the more easily oxidizable reducing agent takes precedence over photo-oxidation of the amine. In the present system it is not feasible to exclude trace amounts of oxygen from the cells, and the addition of glutathione or cysteine in amounts which would be sufficient to inhibit photodynamic inactivation of the tumor cells is injurious to the cells.

It seems probable that, during the photosensitized inactivation of tumor cells, photoautoxidation (involving the cells as the oxidized substrate) is not the only process taking place; in the case of very heavy cell suspensions (about 10^8 cells/ml) and containing 10^{-5} M thionine or methylene blue, photobleaching of the dye is observed. Upon cessation of illumination the leuco dye is re-oxidized by dissolved oxygen. It is probable, therefore, that some or all of the following processes are responsible for the tumor cell inactivation: (*a*) direct photo-oxidation of essential elements of the tumor cells by an excited dye-oxygen complex (photoperoxide) (18); (*b*) oxidation of the tumor cells as a consequence of their electron-donating action to light excited dye molecules; (*c*) oxidation of the tumor cells by hydroxyl radicals formed by (dark) oxidation of leuco dye (17).

The capacity of the dyes to bind to, and to penetrate, the tumor cells may be a necessary but not sufficient condition for their capacity to photosensitize the inactivation of the cells (cf. Table 4): congo red (inactive as a photosensitizer) is bound as strongly to the cells as is thionine (a sensitizer). The values found for the amount of dye binding to Gardner lymphoma cells are in the same range as those reported for the binding of rose bengal to erythrocytes (6×10^{-16} moles bound per cell) (8), and for acriflavine to Ehrlich ascites cells (20×10^{-16} moles bound per cell) (4).

The capacity of cells to exclude certain dyes has been used in measuring their viability (6, 7, 20, 22). When Ehrlich ascites cells are inactivated by depletion or by treatment with poisons or viruses, their capacity to elicit tumor growth was diminished at a greater rate than their capacity to exclude eosine and trypan blue. Pappenheimer (20) reported that, when hematoporphyrin and chlorophyll were used as sensitizers, the photodynamic injury of human lymphocytes greatly increased their permeability to Evans blue and trypan blue. In all the above studies, dye concentrations used were 10^{-2} to 10^{-3} M. We have found no significant difference in the amount of dyes bound to viable or to photoinactivated tumor cells in solutions containing 10^{-5} M dyes. We have no explanation for the fact that in some cases the dyes bound to light-exposed cells are readily eluted with saline. This is true, for example, in the case of methylene blue and neutral red (cf. Table 4). In other cases, e.g., janus green B, the reverse is true, and in yet other instances no differences are noted in dye binding strength between light-exposed cells and dark controls. These observations may, perhaps, be related to the affinities of dyes to specific cell constituents.

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