Modification of Radiosensitivity by Porphyrins

II. Transplanted Rhabdomyosarcoma in Mice

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Summary

Eight days after i.m. injection of rhabdomyosarcoma into the thighs of mice, the tumors were irradiated with 2750 r. Three hr before X-irradiation the mice were given injections i.p. of 0.5 ml of 0.75% sodium bicarbonate alone or of 0.01, 0.05, 0.25, or 1.25 mg of crude "hematoporphyrin," a copper "hematoporphyrin," or a copper "hematoporphyrin" nitrate. The tumor response varied significantly with the dose of porphyrin given: tumors of all 27 mice (100%) who received 0.05 mg of "hematoporphyrin" or its copper complex showed grossly complete regression and did not recur during the following 84-86-day study period, while such regression was seen in only 5 of 126 mice (4%) treated with injection with the other dose levels of porphyrin or with vehicle alone prior to X-irradiation. Normal mice receiving total body X-irradiation showed no similar porphyrin effect, suggesting an increase in the therapeutic ratio (cancer effect/whole body effect) by the 0.05 mg of porphyrin administered. Little, if any, modification of radiosensitivity was produced by the copper "hematoporphyrin" nitrate. Other findings related to the observed porphyrin dose-dependent response curve are discussed.

Introduction

Previous studies (9, 10) in 56 human subjects with a variety of malignant tumors indicated that the speed and extent of regression of only certain types of tumor was probably enhanced by the i.v. injection of "hematoporphyrin" 3 hr before each irradiation of the tumor. Among the patients studied were 3 with rhabdomyosarcoma, generally considered to be relatively radioresistant. Each showed rapid tumor regression during the course of therapy; 1 has no sign of tumor 8 years later, although the other 2 died with distant (untreated) metastases. The present studies were therefore undertaken to determine whether a similar tumor in carefully controlled animal studies would yield unequivocal results regarding the possible potentiation of radiosensitivity of "hematoporphyrin" or some of its derivatives.

During the past 10 years we have studied the effect of a large number of free porphyrins and of metalloporphyrins on X-ray sensitivity of various biologic systems (9). The nature of these porphyrins will be described in detail elsewhere (4), with special reference to the presence of 10 or more different porphyrins in all commercially available preparations of "hematoporphyrin." The best of these contains about 66% hematoporphyrin. Of the compounds tested, some preparations of "hematoporphyrin" (and especially of 2 porphyrin fractions separated from it by countercurrent distribution) and a copper complex of "hematoporphyrin" have been found to be most effective in enhancing radiosensitivity of the transplanted mouse tumors studied. In all cases, however, the modification of radiosensitivity depended markedly on the dose of porphyrin given. The few compounds which enhanced radiosensitivity did so only at very small doses, approximating 0.1-1% of their minimum lethal doses; at higher doses of porphyrin this effect was lost and then often replaced by radioprotection. A copper porphyrin nitrate, which uniquely increases X-ray sensitivity of paramecia by a factor of 30 or more (3), was included in these studies even though it has not been effective when administered prior to X-irradiation of mouse mammary carcinoma4 or of Ehrlich ascites tumor.

Materials and Methods

The "hematoporphyrin" employed was originally pooled from material obtained from several different sources (9). The copper complex was prepared by heating a portion of the free porphyrin with a 3-fold molar excess of copper acetate in 70% acetic acid until disappearance of fluorescence on exposure to near-ultraviolet light. It was precipitated by addition of 3 volumes of water. The copper "hematoporphyrin" nitrate was prepared in glacial acetic acid containing equimolar amounts of copper nitrate, copper acetate, and "hematoporphyrin." It was

1 These studies were performed in the Department of Experimental Medicine and Cancer Research, Hebrew University, Jerusalem, as part of the Sabbatical Year program of S. Schwartz. A report of these studies was submitted by L. Cohen to the faculty of the Medical School in partial fulfillment of the requirements for a Master of Science Degree. The study was supported by grants from the American Cancer Society (T-190A) and the USPHS (GM-K3-14,986 CI-A).

2 Research Career Awardee of USPHS.

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1S. Schwartz, M. Keprios, and R. Toyama, data to be published.
2S. Schwartz, J. Modell, and R. Walters, data to be published.
3S. Schwartz and R. Walters, data to be published.
4S. Schwartz, H. Dinsmore, P. Edmondson, and B. Loudas, data to be published. For brief account see Reference 9.
precipitated with 3 volumes of water immediately after 1st appearance of a greenish color in the solution. The precipitates were filtered, washed copiously with 3% acetic acid and water, and dried. Infrared analysis of the nitrated complex showed it to be mainly a mononitrate.

The rhabdomyosarcoma employed was obtained originally from Klein (4) as an ascites tumor. For several years it was carried by Israeli investigators (7, 8) in RIII mice either as an ascites or solid tumor. The tetraploid nature and other properties of this tumor have been described by Hauschka and Levan (3), by Sachs and Gallily (8), and by Pikovsky and Schlesinger (7). Histologically, the tumor is now quite anaplastic and, in accord with the report of Klein (4) on the repeatedly transplanted tumor, no clear striations are seen with Masson, Van Giesen, or hematoxylin-eosin stains.

For transplantations the solid tumors were minced, passed through a tissue press, and mixed well with an equal amount of physiologic saline. Fractions of 0.05 ml each were then injected i.m. into the right thighs of mice after removing the hair with a fine hair clipper. After 4 generations of such transplantation in Israeli-Swiss mice, 100% "takes" were regularly observed. Without further treatment the mice generally died within 15-25 days. No spontaneous regressions were seen in 80 such untreated mice.

Tumor sizes were measured 8 days after the inoculation and the mice were then treated with different doses as shown in Table 1. Male Israeli-Swiss mice were used in all the studies. In each of the different experiments they were of comparable weight (within ±2 gm), ranging from 18-25 gm at the time of treatment.

Each experiment included a control group of 14-28 mice injected i.p. with 0.5 ml of vehicle alone (0.75% solution of sodium bicarbonate) 3 hr prior to irradiation. From 13 to 14 mice in 4 other groups were each injected similarly with 0.01, 0.05, 0.25, and 1.25 mg of the indicated porphyrin per mouse, respectively. All the tumors received the same dose of X-ray, namely 2750 r (in air). The studies of each porphyrin were done separately, employing for each a different generation of tumor.

For irradiation, the mice were randomized for position within the apparatus shown in Fig. 1, and the exposed leg containing tumor was irradiated at a dose rate of 157.6 r/min. The Picker Vanguard apparatus employed was operated at 280 kv, 20 ma, with 0.5-mm copper and 0.5-mm aluminum filters yielding a half-value layer of 0.65 mm of copper. Tumors were irradiated at a distance of 40 cm from the source. The 6-mm-thick lead shield covering the body of the mice (Fig. 1) was found to transmit less than 1% of the incident dose. A 3-mm-thick lead shield with a hole to transmit the tumor-bearing leg was placed around the inner circumference of the plastic mouse boxes. Following irradiation the mice were randomly distributed in their cages.

Results were evaluated by 3 criteria: (a) survival, (b) tumor size, and (c) the number of mice showing grossly complete tumor regression. Since it was necessary to terminate the experiments after 86, 84, and 62 days after irradiation and treatment with "hematoporphyrin," copper "hematoporphyrin," and copper "hematoporphyrin nitrate, respectively, average survival times have been calculated considering survivors to have lived just to the termination date. Complete tumor regressions (or apparent "cures") are calculated for those tumors which remained nonpalpable and nonmeasurable to the end of the study, or to the time of death.

<table>
<thead>
<tr>
<th>PORPHYRIN</th>
<th>PORPHYRIN DOSE (mg)</th>
<th>NO. OF MICE TREATED</th>
<th>&quot;AVERAGE&quot; SURVIVAL (days)</th>
<th>SUMMARY AS OF DAYS 86 AND 84</th>
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<tbody>
<tr>
<td></td>
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<td></td>
<td>No. of survivors</td>
</tr>
<tr>
<td>1. Hemato-</td>
<td>0</td>
<td>28</td>
<td>36</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>13</td>
<td>67</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>14</td>
<td>85</td>
<td>13</td>
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<tr>
<td></td>
<td>0.25</td>
<td>14</td>
<td>44</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>14</td>
<td>41</td>
<td>0</td>
</tr>
<tr>
<td>2. Cu-Hemato</td>
<td>0</td>
<td>14</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>14</td>
<td>74</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>13</td>
<td>82</td>
<td>12</td>
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<tr>
<td></td>
<td>0.25</td>
<td>14</td>
<td>62</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>14</td>
<td>49</td>
<td>5</td>
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</table>

As of termination date of experiment (86 and 84 days, respectively).
Although the 0.01-mg dose of either porphyrin prolonged average survival times, the apparent potentiating effect produced was insufficient to yield grossly complete regression of the tumor. At the 0.05-mg dose level, however, all 27 mice showed grossly complete disappearance of tumor. Two of these mice, with no gross sign of tumor, died shortly before termination of the study. (Two mice in the other 8 groups also died of unknown cause with no observable evidence of tumor.)

The time relationship of the tumor growth response is summarized in Table 2, comparing the optimum results obtained with 0.05 mg of either porphyrin with those seen in mice receiving vehicle alone or 1.25 mg of porphyrin prior to irradiation. By Day 36, tumor was not palpable or measurable in any of the 27 mice receiving the 0.05-mg dose. The “hematoporphyrin” appeared to be somewhat more effective as regards both the greater rapidity of tumor regression at the 0.05-mg dose level, and the nature of the response at the 1.25-mg dose level. At the latter dose level, the copper complex appeared to be radioprotective. (The values listed in the groups which received vehicle alone or a dose of 1.25 mg of either porphyrin, are of course influenced by the increasing mortality seen in these groups after Day 22; at least half the mice in each of these 4 groups (generally with large tumors) had died by Day 43. Since values are based only on surviving mice, the values listed are minimum ones in terms of each total treated group. There were no deaths during this period in the 0.05-mg-treated groups.)

### Table 2

**Average Tumor Diameters following X-irradiation in Mice Given Injections of 0, 0.05, and 1.25 mg of **“**Hematoporphyrin**” or of Copper **“**Hematoporphyrin**”

<table>
<thead>
<tr>
<th>X-ray dose of 2750 r (in air) to tumor in all cases. Vehicle = 0.5 ml of 0.75% NaHCO₃. **“<strong>Hematoporphyrin</strong>” administered in Experiment 1; copper **“<strong>hematoporphyrin</strong>” in Experiment 2. No. of mice per group as given in Table 1.</th>
</tr>
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<tbody>
<tr>
<td>TIME AFTER TREATMENT (DAYS)</td>
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<td></td>
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<tr>
<td></td>
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<tr>
<td>0</td>
</tr>
<tr>
<td>8</td>
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<tr>
<td>15</td>
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<tr>
<td>22</td>
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<td>29</td>
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<tr>
<td>36</td>
</tr>
<tr>
<td>43</td>
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</tbody>
</table>

* Difference between tumor and tumor-free thighs.
* Only 1 tumor still palpable.
* Ten tumors still palpable.

B. Total Body X-irradiation

In 2 separate experiments, normal mice were exposed to 400 r total body X-irradiation 1 hr before or 3 hr after i.p. injection of 0.5 ml of 0.75% sodium bicarbonate alone or with 0.03, 0.05, 0.2, 0.8, or 1.5 mg of “hematoporphyrin.” In 2 other experiments done at a later date, they were similarly given injections of the same vehicle alone or of 0.05, 0.25, or 1.25 mg of copper “hematoporphyrin” before or after irradiation. Because of the small number of mice in each group and because distinct differences were observed only in groups receiving small vs. large doses of porphyrin, results have been pooled as shown in Chart 1. It is seen that the cumulative mortality curve of the 80 mice which received 0.03–0.25 mg of either porphyrin was essentially the same as that of the vehicle alone. The earlier deaths in the 60 mice which received 0.8–1.5 mg of porphyrin suggests a possible potentiating effect of this large dose. (The minimum lethal dose of the copper complex in these mice was 4–5 mg, while that of the “hematoporphyrin” was approximately twice this amount.)
Discussion

The present study clearly shows that small doses of the crude "hematoporphyrin" and copper "hematoporphyrin" preparations injected prior to X-irradiation significantly enhanced the response of transplanted rhabdomyosarcoma in mice under the conditions employed. This is the 1st time we have found a variation from 0 to 100% in the apparent "cure" rate depending upon the porphyrin dose employed in a transplanted mouse tumor irradiated in vivo, although such "all or none" effects are readily demonstrable in paraecia or in Ehrlich ascites tumor cells irradiated in vitro and then reinjected into mice.  

Since, in these latter systems as well as in mice with transplanted mammary carcinoma significant enhancement is observed only at low dose levels of porphyrin, the lack of radiosensitization by "hematoporphyrin" and copper "hematoporphyrin" reported by others (1) is readily understood. In these negative studies the authors administered 3–6 doses of 0.6–1.5 mg of these porphyrins to mice prior to X-irradiation of their transplanted tumor (Sarcoma 180 and/or neuroblastoma c-1300). While the rationale of employing maximum tolerated doses may be applicable to the study of chemotherapeutic responses, it is certainly not applicable to the evaluation of the effect of porphyrins as modifiers of tumor radiosensitivity. It is also to be emphasized that the composition of commercially available "hematoporphyrin" is inconstant. Although all the samples tested here have shown a dose-dependent effect, the magnitude of the effect and the optimum dose vary with different tumors. Thus, 0.01 mg of "hematoporphyrin" or copper "hematoporphyrin" is consistently more effective than 0.05 mg in irradiated mouse mammary carcinoma, and 1 fraction isolated from crude "hematoporphyrin" is more effective at a dose of 0.1–0.3 mg in the same tumor.  

It is obviously important to determine whether or not the potentiating dose of porphyrin (approximately 0.05 mg in the present study) exerts a differential effect on irradiated tumor and nontumor tissues. In the absence of such an increase in the "therapeutic ratio," increased response in the tumors alone would be interesting, but of no potential clinical significance. Since such small doses of porphyrin administered to normal mice receiving total body X-irradiation did not modify their mortality curve, it would seem that they do indeed increase the therapeutic ratio. Obviously, too, it would be desirable to make such comparisons in tumors and individual normal tissues such as skin, white blood cells, etc. Studies of tumors irradiated with various doses of X-ray would also be desirable to determine the % enhancing effect of the porphyrins. It is felt, however, that studies of these and other comparisons should be postured until the nature of the porphyrin dose response phenomenon is better understood and controlled.  

The mechanisms of action of the porphyrin in X-irradiated systems are unknown. As described elsewhere (5), large doses of porphyrin administered to normal mice result in decreased total body oxygen consumption, hemostasis, and, presumably, tissue anoxia. This anoxia might explain the loss of potentiating (or increasing protective effect) observed in mouse tumors irradiated after large doses of porphyrin. It cannot, however, explain the completely similar results observed in paraecia and in Ehrlich ascites tumor cells irradiated in vitro. It is tempting to postulate that the porphyrin may significantly affect free radicals or energy transfer mechanisms related to the primary events which follow X-irradiation. The highly resonating structure of the porphyrin ring and its selective affinity for proteins would be consistent with such an hypothesis, as is the finding that radiation-enhancing effects in paraecia are found only when porphyrin is present during the irradiation period. Since, in the latter system, radioprotection is observed even if porphyrin is added only after irradiation, other mechanisms must operate here. Inhibiting or enhancing effects of porphyrins on various enzyme systems (11) may also be significant.  

Granick and Gilder (2) have shown that "hematoporphyrin" acts as a metabolite antagonist toward protoporphyrin in Haemophilus influenzae, the latter porphyrin being required for the growth of the organism. We have also found the reverse effect, namely protection by protoporphyrin of normal mice administered another otherwise lethal dose of "hematoporphyrin" (6). The possibility that altered metabolism of porphyrin-containing enzymes may be involved in the tumor responses observed remains to be determined. It may also be of interest that protoporphyrin and protohemin exert significant protective effects on irradiated mouse mammary carcinoma.  

Different amounts and types of porphyrin have been administered to mice bearing various types of tumor without additional irradiation or other therapy. In these studies, the porphyrin alone has not affected tumor growth except where repeated large doses have resulted in decreased food intake and loss of body weight. One can therefore assume that the effects reported herein were potentiating and not additive.  

Other aspects of this problem will be considered in the unpublished studies cited in the footnotes.

Acknowledgments

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References


7 R. Fabiny, S. Schwartz, and L. Wattenberg, data to be published.

**Fig. 1.** Mouse holder used for X-irradiation. A, plastic boxes used for X-irradiation of tumor-bearing leg or of total body. B, added ead shield exposing only the tumor-bearing leg.
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