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In Vivo Experimental Studies on the Role of Free Radicals in Photodynamic Therapy. II. Photodynamic Effect on Free Radical Concentration in Mice Tumors Measured by ESR Spectroscopy

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Changes in free radical concentrations of solid tumors of mice after photodynamic treatment using Photofrin II sensitizer has been measured by ESR spectroscopy. Decrease in the free radical concentrations by 46% in S_{180} and by 35% in P_{388} tumors induced photodynamically was found. As expected, the effect was proportional to the steady state concentrations of the free radicals in the malignant cells of mice prior to photodynamic treatment if comparing either different types of tumors or data referring to various stages of the development of identical types of tumors. Results support the contribution of the interactions between triplet excited sensitizer molecules and native free radicals to photodynamic effects as assumed earlier.

Photodynamic Therapy (PDT) is a relatively new modality for the treatment of malignant cells. The mechanism of the primary sensitization steps could be of essential importance concerning the efficiency of the treatment. It has been suggested by us [1–2] that in the course of photosensitization under *in vivo* conditions excited triplet sensitizer molecules, (^{3}PS), might interact directly with native free radicals, (^{2}Rad$^\bullet$), generated in live tissues as a result of biochemical processes. It should be mentioned that studies in chemical model systems yielded quantitative parameters for this process using laser flash photolysis and kinetic ESR spectroscopic techniques [3–4]. Thus the triplet-doublet interaction:

$$^3\text{PS} + {}^2\text{Rad}^\bullet \xrightarrow{k_1} \text{products} \qquad [1]$$

depending on the conditions can compete with process which generates highly reactive oxygen species:

$$^3\text{PS} + {}^3\text{O}_2 \xrightarrow{k_2} {}^1\text{PS} + {}^1\text{O}_2 \qquad [2]$$

assumed earlier as a main route for photodynamic effects, (PDE), [5–7]. If considering above assumption kinetically, it seems obvious that results of the competition mentioned above will depend on the relative concentrations of oxygen and of free radicals in the tumor tissue. The precondition for the interaction (1) is a minimal concentration of Rad$^\bullet$ in the tumor cells during PDT. In order to prove the existence of such minimal concentrations of native free radicals, a method has been elaborated and applied to measure the steady state concentrations of free radicals in tissues as described in Part I. of this series [8]. The next step in finding further supporting experimental evidence for the triplet-doublet interaction as an actually contributing mechanism to the overall PDE, was the study of the *changes* in [Rad$^\bullet$] during PDT. Results are summarized in the present paper.

MATERIALS AND METHODS

Chemicals. Photofrin II (Quadra Logic Technologies Inc. Raritan, NJ) was obtained as an aqueous solution at a concentration of 2.5 mg/ml and stored at −20°C. Prior to injection it was thawed, allowed to come to room temperature and

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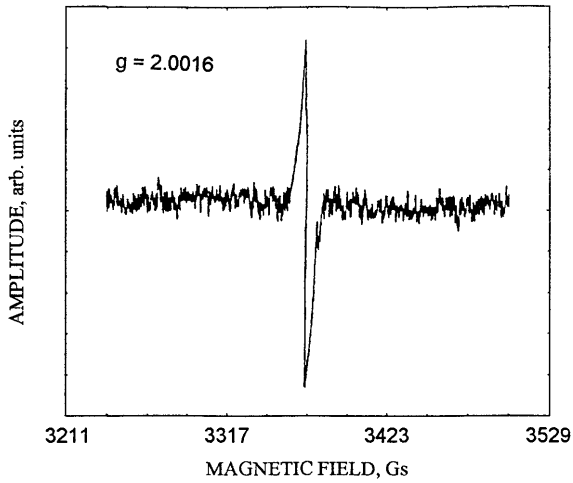

FIG. 1. ESR spectrum of the S_{180} solidtumor. Center field: 3341 ± 150 Gs; modulation: 2 Gs; sweep time: 1 min; time constant: 0.1 s.

used after dilution by sterile 0.9% Sodium Chloratum (Biogal) up to 1 mg/ml. Nembutal (Abbott Laboratories) was used for anesthesia of mice without purification.

Animals and tumors. Tumor bearing BDF_1 cross-breed, specified pathogen free mice were received from the National Institute of Oncology, weighed 20–22 g. We used two different tumor lines: S_{180} sarcoma applied in our experiments derive from Chester Beatty Cancer Research Institute, London, UK, 1968. P_{388} lymphoid leuchaemia was obtained from Wodinsky, J. Arthur Little and Co. Cambridge, Ma., USA. Both of them are s.c. growing solid tumors in interscapular region of mice. The tumor volumes were calculated using the following expression:

$$V = \pi/6\ (L \times D^2)$$

where L is the longer and D is the shorter diameter of the tumors measured with calliper square. Results described in this work correspond to about 220 mice.

Light illumination of the tumor. Illumination of the tumors was carried out 24 hours after i.p. injection of 15 mg/kg

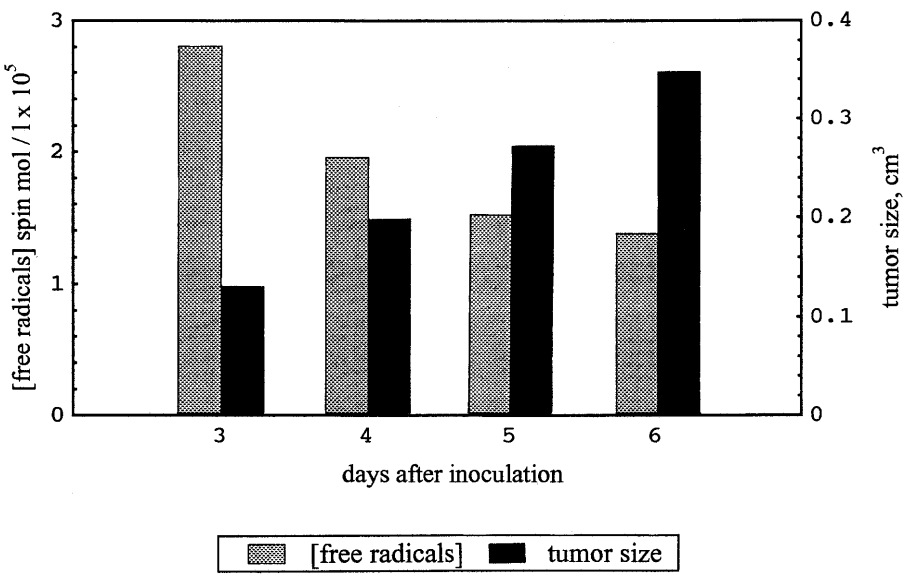

FIG. 2. Changes of free radical concentrations and growth of S_{180} tumor between the 3rd and 6th days after inoculation (in the case of free radicals between the 3rd and 4th days $p < 0.001$; between 4th and 5th $p < 0.05$).

TABLE 1
Effect of Photodynamic Treatment (15 mg/kg of Photofrin II; 150 J/cm^2 Light Dose) on the Concentrations of Free Radicals of S_{180} Tumor. Values are Means ± SE

N (mice)	Control	PDT	Effect (%)
	[free radicals] spin mol/1 × 10^5		
20	1.85 ± 0.33	0.98 ± 0.17	47
10	2.10 ± 0.45	1.07 ± 0.17	49
20	1.97 ± 0.21	1.15 ± 0.18	42
total: 50	av: 1.97 ± 0.09	1.07 ± 0.06	46 ± 3

Photofrin II by a PTL PENTA 250 W lamp at wavelengths 610–654 nm by using filters under general anesthesia of mice. The fluence rate was 168 mW/cm^2 and the total light dose was 150 J/cm^2 for 15 minutes. These values were controlled by an IL 1350 Radiometer/Photometer.

Preparation of samples and ESR measurements. The ESR spectrometer was a computer controlled equipment constructed and built by the Institute of Chemical Physics of the Russian Academy of Sciences with a sensitivity of about 10^{-6} spin mol 1^{-1}. Tissue samples have been prepared 5 mins after light illumination by freezing the whole tumor. Measurements were performed in a quartz Dewar filled up with liquid nitrogen. Fig. 1. shows the typical ESR spectra of S_{180} tumor. The g-factors have been determined using MnO/Mn standard. After signal accumulation (25–50 sweeps) the spectra using their second integrals yielded the steady state concentrations of free radicals by comparison with solutions of the stable free radical 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO) as standard.

RESULTS AND DISCUSSION

Preliminary experiments were performed in order to determine the relation between the kinetics of growth and the free radical contents in S_{180} tumor. Fig. 2. shows the decrease in the steady state concentrations of free radicals between the 3rd and 6th day after inoculation. The same figure demonstrates the even increase in the volume of the S_{180} tumors during this time period. Results obtained for tumor growth are in accordance with literature data [9–11] in which growth index has been found to correlate roughly to the tumor size expressed in cm^3 and in this time period a linear plot of proliferation kinetics of S_{180} tumors has been observed. As it can be seen, the changes in the free radical concentrations and tumor volumes show opposite tendencies. These results enabled us to choose the optimal day for PDT treatment. The fourth day was chosen when the tumor already has well measurable volume and free radical concentrations are still high enough.

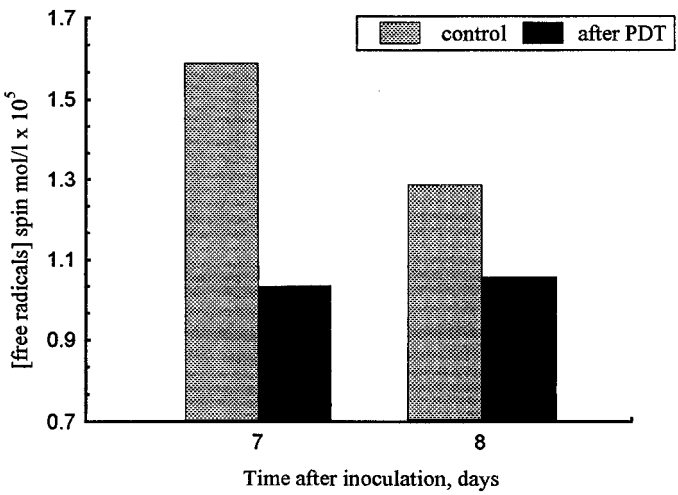

FIG. 3. Comparison of the efficiency of photodynamic treatment on P_{388} tumor between the 7th and 8th days after inoculation ($p < 0.05$).

TABLE 2
Effect of Photodynamic Treatment (15 mg/kg of Photofrin II; 150 J/cm^2 Light Dose) on the Concentrations of Free Radicals of P_{388} Tumor. Data are Expressed as means ± SE

N (mice)	Control	PDT	Effect (%)
	[free radicals] spin mol/1 × 10^5		
10	1.44 ± 0.11	0.92 ± 0.11	36
11	1.49 ± 0.05	0.98 ± 0.03	34
10	1.70 ± 0.17	1.13 ± 0.18	34
10	1.58 ± 0.43	1.03 ± 0.15	35
total: 41	av: 1.66 ± 0.13	1.08 ± 0.08	35 ± 1

Table 1 summarizes the results of PDT treatments of S_{180} tumor. Doses used in our experiments have been curative in a large number of animals [12]. In each series the free radical concentrations of the treated groups was compared with the corresponding values of the untreated control groups. Definite decrease has been found in the free radical concentrations indicating a PDT-induced effect. Average value of this effect for S_{180} tumor was 46%.

In order to compare the efficiency of PDT treatment on different tumor models experiments were performed also with P_{388} tumor which has been used for prescreening anticancer drugs since 1975 [13,14].

In Part I. [1] it has been shown that the maximal concentrations of free radicals was reached on the 7th day after inoculation by P_{388} tumor. Because it is a less quickly growing tumor its volume on the 7th day equals approximately the volume of S_{180} tumors on the 4th day after inoculation of the latter, being about 0.1 cm^3. In the present work it was established that the 7th day is the most suitable for PDT treatment of P_{388} tumors since one day later when the free radical concentrations start to diminish the therapy-induced effect has decreased by 17% as shown in Fig. 3.

Table 2 shows that in the case of the treatment of P_{388} tumors an average 35% decrease in radical concentrations was observed. It is interesting to note that the steady state concentrations of the free radicals in untreated control groups of tumor P_{388} was lower by 16% as compared with S_{180} tumor. The following comparison of Table 1. and 2. shows that the difference in the average PDT-induced effects between the two tumor models was 11%.

It is suggested that this difference can be explained by the increased level of free radicals in S_{180} tumor. Namely, it has been derived already [1] that processes (1) and (2) give the following expression:{equa 3 here}and since $k_1/k_2 \sim 1 - 4$ the rates of the photodynamic effects depend basically on the free radical/oxygen ratios in the malignant tissue. If assuming that the concentrations $[O_2]$ have not differed in the tumor tissues of the mice, the effects exerted by direct interactions between ^{3}PS and ^{2}Rad$^\bullet$ vary depending on [^{2}Rad$^\bullet$].

Consequently, comparing different types of tumors, the PDE should be larger in those cases where [Rad$^\bullet$] is greater. Similarly, comparing tumors of the same type but at varying stages of development of the tumor, the same is expected. Although such comparison at present is limited to two types of tumors, but to support it, more experiments will be carried out in the future using tumor models with different steady state levels of free radicals.

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