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In Vivo Experimental Studies on the Role of Free Radicals in Photodynamic Therapy

III. Photodynamic Effect on Free Radicals Generated in Cell Cultures

Dezső Gál, Tamás Kriska, and Elena Maltseva[1]
Central Research Institute for Chemistry, Hungarian Academy of Sciences, P.O. Box 17, H-1525 Budapest, Hungary

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The effect of excited Photofrin II molecules on the zymosan stimulated and luminol dependent chemiluminescence (CL) of macrophages, known as respiratory burst, has been studied. Excitation was carried out by light irradiation in the vicinity of the maximal CL by varying light doses. In separate experiments potassium salt of 3,5-di-*tert*-butyl-4-hydroxyphenyl propionic acid radical inhibitor was added to the system and its consumption measured spectroscopically. Based on the experimental results the decrease in the steady state radical concentrations under the influence of radical inhibitor and of triplet sensitizer generated *in situ* has been calculated. It has been established that the corresponding photodynamic effect is an inhibition-like process proceeding by triplet–doublet interactions.

In the previous papers of this series [1-2] we have referred to the possibility to measure directly the actual steady state concentrations of free radicals generated in tumor cells of mice using frozen samples and their reduction due to the interactions with excited triplet sensitizer molecules. These experiments intended to support the assumption that such triplet-doublet interactions might play important role in photodynamic effects under *in vivo* conditions [3] and might give an explanation to the difficulties to determine singlet oxygen *in vivo* [4] in the presence of excited sensitizer.

Although results were in favour of the mechanism suggested, the complexity of the system made it difficult to derive unambiguous conclusions, since the strong decrease in the steady state concentrations of native free radicals in the tumor cells in the presence of excited sensitizer might be due to the effect exerted both on precursors generating the radicals and/or on the radicals themselves.

In order to separate these two types of effects it seemed expedient to apply a "simpler" model system and a more sensitive method for such studies. In the present paper the effect of triplet excited sensitizer molecules on the zymosan induced and luminol dependent chemiluminescence (CL) of cell cultures will be described. Details of CL observed in the absence of photosensitizer were submitted for publication elsewhere [5].

MATERIALS AND METHODS

Chemicals. Photofrin II (Quadra Logic Technologies Inc.) was obtained as an aqueous solution at a concentration of 2.5 mg/ml and stored at −20°C. Before use it was diluted to 12.5 μg/ml. Potassium salt of 3,5 di-*tert*-butyl-4-hydroxyphenyl propionic acid (Phenozan) was synthesized in the Institute of Chemical Physics, RAS and used without further purification. Luminol (Sigma) was applied in DMSO stock solution in concentrations of 100 mM. Zymosan (Sigma) was used without further purification and a stock solution of 10 mg/ml was prepared.

Cell culture. Elicited macrophages have been harvested from male DBA/2 mice (20-22g) by peritoneal lavage using RPMI 1640 medium (Sigma) containing 10% fetal calf serum (Gibco) and 5E/ml heparin. To obtain elicited macrophages mice were injected i.p. with 1 ml sterile 10% sodium caseinat (Sigma) prepared in 0.9% NaCl. Cells were incubated at 37°C for 2 hours then the complete medium was exchanged to serum and heparin free medium. During experiments cell cultures were kept at 37°C.

CL measurement. Before the experiments the adherent cells were washed twice with Hank's balanced salt solution (Sigma, pH 7.2). A 5 ml mixture containing 2×10^6 cells in monolayer, 0.2 % bovine serum albumin, 0.2 % glucose and 1.25μg/ml Photofrin II did not yield "native" CL. Therefore, stimulation was carried out by the addition of 0.5 mg/ml zymosan. Unfortunately, either the stimulated CL was negligibly low or the interactions of the radicals generated by stimulation have not resulted in CL, measurable CL was achieved only if luminol was added to the mixture (100 μM). Preliminary experiments have shown that Photofrin II had no effect on the CL signal.

Measurements have been performed in a specially built, computer aided chemiluminescence apparatus in which the cavity was equipped with a slot for irradiation of the sample. Irradiation was carried out by a PTL PENTA 250 W lamp in a wavelength region 610 - 650 nm using filters. During irradiation the 9658 EMI (S-20)

[1] Present address: Institute for Biochemical Physics, Russian Academy of Sciences, Moscow, Russia.

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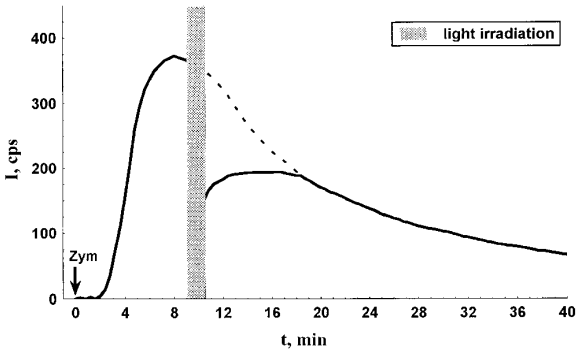

FIG. 1. Effect of excited Photofrin II on the chemiluminescence of macrophages (2×10^6 cells) stimulated by 0.5 mg/ml zymosan in the presence of 10^{-4} M luminol and 1.25 μg/ml Photofrin II (dotted line refers to experiments without excitation).

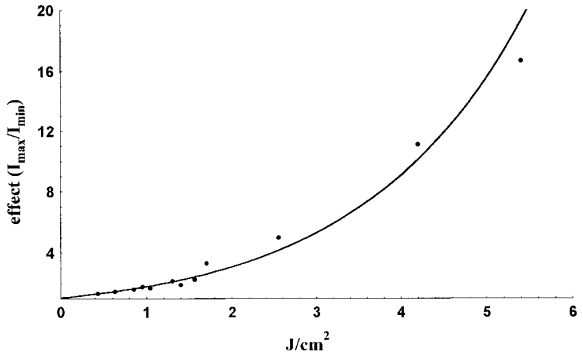

FIG. 2. Zymosan stimulated and luminol dependent chemiluminescence intensity of macrophages against light dose absorbed by Photofrin II sensitizer.

type photomultiplier was automatically closed, using a tightly fitted metal window to avoid any damage caused by the strong light used for the excitation of the sensitizer. The total efficiency of the CL analysis measured by the luminol method [6] was 3.40 ± 0.2 %.

This means, however, that during the irradiation periods previously programmed and varied according to requirements, CL signal was not registered.

In order to obtain reliable data, excitation of the sensitizer has been carried out at high CL signal intensities, that is, in the vicinity of the CL maxima.

In a few experimental run instead of Photofrin II, a free radical inhibitor developed for biological systems, Phenozan, was added to the reacting mixture at the same time when in the standard experiments (that is, in the presence of Photofrin II) irradiation would have been commenced.

Analysis. In addition to the CL measurements, consumption of Phenozan and of luminol have been followed by their UV spectra in the range 252 - 298 nm and at 350 nm, respectively, in samples taken from the reaction mixture.

RESULTS AND DISCUSSION

The zymosan stimulated, luminol dependent CL of cell cultures, called *respiratory burst* [7] can be represented by an unsymmetric bell-shape curve. If in the presence of Photofrin II. the mixture is irradiated and the photosensitizer excited, after irradiation (triplet excited sesitizer molecules do not exist anymore in the system due to their short lifetime) the CL signal starts to increase from a much lower value than observed before irradiation. The uneffected and affected curves of the respiratory burst are represented by Fig. 1. where the dotted line gives the measured values without irradiation. As it can be seen from Fig. 1. the increase after irradiation is slow and reaches the unaffected CL signal at a later stage.

The effect of the light dose absorbed is shown by Fig.2. where the ratios of the CL signal intensities at the start (I_{max}) and the end of irradiation (I_{min}) are given against the light doses and represent an exponential increase.

It was mentioned already that during the irradiation period CL can not be registered because of the experimental arrangement itself. In order to "simulate" the process during this time period, in separate experiments Phenozan was added to the system at I_{max} without performing irradiation. Corresponding results are shown by the dotted line in Fig.3. In this case the inhibitor, in decreasing amounts though, remains in the system and thus radical "scavenging" proceeds continuously in contrast to the experiments carried out in the presence of excited sensitizer molecules, when after irradiation the active species are discarded from the system. Consequently, addition of Phenozan does not result in the increase of the CL signal after its sudden fall but follows a very slow approximation to the unaffected values determined by the rate of consumption of the Phenozan molecules. Furthermore, since the inhibitor exerts its effect immediately after its addition to the system, it can be assumed that the inhibition takes place in the solution before its cellular uptake.

Comparison of Figs. 1. and 3. indicates that triplet excited sensitizer molecules act as "inhibitors" decreasing the steady state concentrations of radicals generated in the system. Therefore, calculations have been performed according to this assumption.

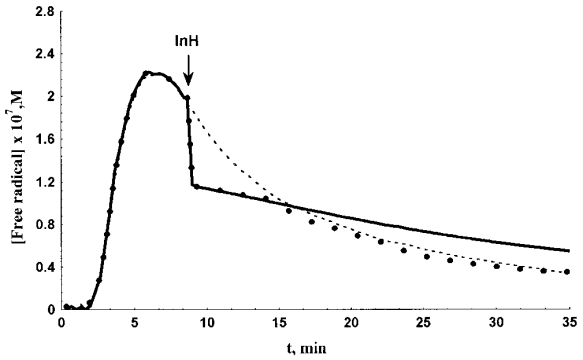

FIG. 3. Experimental (points) and calculated (solid line) concentrations of free radicals in the presence of inhibitor (addition of inhibitor shown by an arrow). Dotted line refers to uninhibited experiment.

Corresponding to literature data [8-9] and to the negligible consumption of luminol during the whole procedure, measured analytically by us, in first approximation:

$$[Rad^{\bullet}]_{min} = [Rad^{\bullet}]_{max} \frac{I_{max}}{I_{min}} \quad (1)$$

Independent experiments have shown [5] that under present conditions $[Rad^{\bullet}]_{max} \sim 2 \times 10^{-7}$ M and using this value the decrease in the radical concentrations in the presence of triplet photosensitizer molecules at varying light doses is given in Table 1.

In the presence of inhibitor, following its addition at I_{max}, the rate of accumulation of free radicals can be represented by the equation:

$$\frac{d[Rad^{\bullet}]}{dt} \sim w(t) - k_t[Rad^{\bullet}]^2 - k[Rad^{\bullet}][Lum] - k_{inh}[Rad^{\bullet}][InH] \quad (2)$$

where w(t) is the total rate of generation of radicals in the system and k_t the rate constant of the termination of radicals e.g. in bimolecular processes, while k_{inh} corresponds to the rate constant of the interaction between radicals and Phenozan. Numerical integration using literature data for $k_{inh} \sim 2 \times 10^4$ M^{-1} s^{-1}, [10], yielded the $[Rad^{\bullet}] \rightarrow t$ curve given in Fig. 3. together with measured values.

Fitting can be considered good.

Similar procedure for systems in the presence of excited sensitizer resulted in less satisfactory results. It was assumed that an "inhibitor-like" compound (excited sensitizer) was present *temporarily* in the system for the time period of irradiation and discarded afterwards when the overall process proceeds again "uninhibited". In this case for the irradiation period instead of equ. 5. we can use:

$$\frac{d[Rad^{\bullet}]}{dt} \sim w(t) - k_t[Rad^{\bullet}]^2 - k[Rad^{\bullet}][Lum] - k_{TD}[Rad^{\bullet}][^3PS^*] \quad (3)$$

where k_{TD} refers to the rate constant of the process between free radicals and triplet sensitizer molecules and was taken from literature sources being $\sim$ (6 - 8) $\times 10^9$ M^{-1} s^{-1} [11-12]. Numerical integration yielded a value for the steady state concentration of triplet sensitizer molecules of about 10^{-9} M by an order of magnitude higher than expected based on earlier calculations [13].

Comparison of calculated and experimental results in Fig.4. shows serious contradiction.

Namely, calculations result, rationally, in the immediate increase of the CL-signal to its unaffected values after irradiation has been stopped, while experimentally a slow increase is observed.

This discrepancy indicates that the decrease in the radical concentrations is not due *exclusively* to the triplet-doublet interactions, but partially also to the effects of the excited sensitizer on the generation of radicals or on the precursors of the free radicals as suggested by other authors [14]. This could also explain the higher value obtained for $[^3PS^*]$. On the other hand, the fact that after the slow increase in the CL signal, observed experimentally, registered values *reach* the unaffected curve, supports the suggestion that the additional effect is not irreversible.

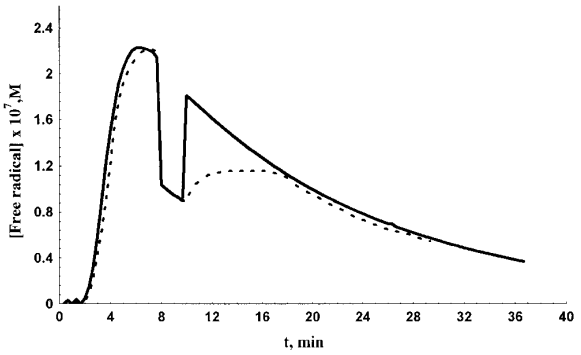

FIG. 4. Experimental (dotted line) and calculated (solid line) concentrations of free radicals in the presence of photosensitizer excited temporarily (excitation experimental data are not available).

TABLE 1

Decrease of the Concentrations of Free Radicals in the Presence of Photofrin II Sensitizer Excited by Varying Light Doses

Light dose (J/cm^2)	$\Delta[Rad^{\bullet}] \times 10^{-8}$ (M)
0.43	4.5
0.63	5.7
0.85	6.8
1.00	7.6
1.30	9.7
1.56	11.3
1.70	12.2
2.55	14.5
4.20	16.4
5.40	17.1

Note. [Photofrin II] = 1.25 μg/ml; cell number = 2×10^6.

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