Vol. 90, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 27, 1979 Pages 460-465

PHOTODYNAMIC MUTAGENICITY IN MAMMALIAN CELLS

Nachman Gruener* and Michael P. Lockwood

Tulane University
School of Public Health and Tropical Medicine
Department of Environmental Health Sciences
New Orleans, Louisiana 70112

Received August 10,1979

SUMMARY

Photoactivation of bound rose bengal in the presence of oxygen causes mutations in chinese hamster embryo cells. Visible light by itself can also cause a slight increase in mutation frequency. Both reactions are amplified by deuterium oxide. Singlet oxygen reactive compounds, β-carotene and 1,3-diphenylisobenzofuran diminish the toxic and mutagenic effects. 12-O-tetradecanoyl-phorbol-13-acetate, a potent tumor promoter, increases the number of mutants induced by the photodynamic action. The enhancement of mutagenesis by deuterium oxide and its reduction by specific singlet oxygen antagonists suggest that this active oxygen species is the direct mutagen.

Active oxygen species such as singlet oxygen, superoxide anions and hydroxyl radicals are known to be formed in normal biological processes (1,2), and are toxic to various biological systems in vivo or in vitro (3,4). Singlet oxygen ($^1O_2$) has been shown to be the mediator of many photosensitized oxidations (5). In such reactions a photosensitizer (Sen) is excited by the absorbtion of light, and is transformed to the relatively short-lived singlet state (^{1}Sen). ^{1}Sen undergoes spin inversion to the relatively long-lived species, ^{3}Sen, which is then quenched by ground state triplet molecular oxygen ($^3O_2$). This energy transfer reaction results in the formation of singlet oxygen ($^1O_2$) and ground state sensitizer (Sen).

$$\text{Sen} \xrightarrow{h\nu} {}^1\text{Sen}$$

$${}^1\text{Sen} \longrightarrow {}^3\text{Sen}$$

$${}^3\text{Sen} + {}^3O_2 \longrightarrow \text{Sen} + {}^1O_2$$

*To whom correspondence should be addressed.

Abbreviations: PBS, phosphate buffered saline (pH, 7.4); DPBF, 1,3-diphenylisobenzofuran; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; DMEM, Dulbecco's Modified Minimum Essential Medium; IFBS, inactivated fetal calf serum (56°C, 30 min.); Sen, photosensitizer.

0006-291X/79/180460-06$01.00/0

The sensitizer is a photoreactive material whose ΔE between the triplet and ground state exceeds 22 Kcal/mole., the ΔE between oxygen's $^1O_2$ and $^3O_2$ states. The excited $^1O_2$ is then available for reactions with suitable oxidizable molecules.

In the following paper we will present evidence that singlet oxygen can induce mutations in mammalian cells.

MATERIALS AND METHODS

Chemicals. Chemicals and cell culture reagents were purchased as follows: Sensitox II, (the photoreactive rose bengal immobilized on polystyrene beads) from Hydron Laboratories (New Brunswick, N.J.); β-carotene, deuterium oxide (D_2O), and ouabain from Sigma Chemicals (St. Louis, Mo.). 1,3-diphenylisobenzofuran from Aldrich (Milwaukee, Wis.); and all cell culture media and reagents from GIBCO (Grand Island, N.Y.). 12-O-tetradecanoyl-phorbol-13-acetate was kindly supplied by R.K. Boutwell.

Cell Culture. Chinese hamster embryonic lung cells (V79) were cultured in DMEM (5) supplemented with 10% IFBS. Cells were maintained at 37°C in a 10% CO_2 atmosphere. Subcultures were performed using 0.25% trypsin in 0.01% ethylenediaminetetraacetic acid.

Mutagenesis Assays. Ouabain resistant mutants were isolated from V79 cells which had been exposed to the bound rose bengal in the presence of light. Briefly, $3X10^6$ V79 cells were plated in 60 mm plastic petri dishes (Falcon) 16 h prior to treatment, in 4 ml DMEM plus 10% IFBS. Cells were washed and exposed to 100 mg/plate of the bound rose bengal suspended in 2 ml PBS at 24°C in the presence and absence of light. The light source was a 15 watt fluorescent lamp (Buchler Instruments) approximately 20 cm above the plates, yielding 150 ft-candles at the cell surface. The light caused no increase in temperature under these conditions. After exposure, the plates were washed three times with PBS. Each plate was then trypsinized, counted and replated in 10 plates with $3X10^5$ cells per plate in 4 ml DMEM plus 10% IFBS. One hundred cells were also seeded in each of 4 plates per point for determination of cytotoxic effect. Forty-eight hours after treatment, ouabain (final concentration, 1mM) was added to plates being assayed for mutagenicity. Seven days after plating, cytotoxicity was determined by cloning efficiency in those plates previously seeded with 100 cells. Cells were fixed in methanol and stained with Giemsa. Plates being assayed for ouabain-resistant mutants were fixed and stained 14 days after treatment. Ouabain resistant mutants isolated at random and grown for one month without ouabain were found to be resistant to different concentrations up to 1mM.

β-carotene, DPBF and TPA stock solutions were prepared in acetone immediately before testing. Acetone levels did not exceed 0.02% per plate. When the effects of D_2O were examined, it was substituted for the H_2O in PBS.

RESULTS AND DISCUSSION

Data presented in Fig. 1a shows that illumination of V79 cells in PBS induced a slight increase in the number of mutants above the background. The

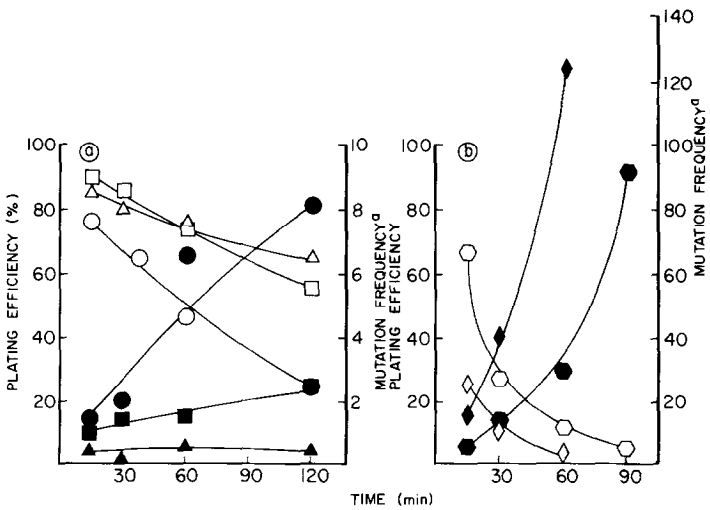

Fig. 1. Time course of photodynamic mutagenicity. Assays were performed as described under Materials and Methods for the following conditions: D_2O in the dark (△ ▲), PBS in light (□ ■), D_2O in light (○ ●), Sen in light (⬡ ⬢), Sen and D_2O in light (◇ ◆). Open symbols indicate plating efficiency and closed symbols indicate mutation frequency.
[a]Mutation frequency is expressed as number of mutants per 10^6 surviving cells.

exchanged of H_2O with D_2O enhanced cell lethality and mutagenicity in the presence of light. D_2O in the dark is somehow toxic but not mutagenic. Others have also found that extended periods of exposure to visible light in H_2O medium leads to an increase in the mutation frequency of mammalian cells (7,8). The enhancement effect of D_2O suggests that $^1O_2$ is involved in the toxic mechanism. Light may excite intracellular photosensitive compounds such as flavins which can be scavenged by ground state oxygen to produce $^1O_2$.

Previous studies have shown that irradiation of soluble photosensitive chemicals can cause mutagenicity in bacteria (9,10). The possibility that $^1O_2$ or a dye derivative or both are responsible for the DNA damage could be implied. To test unequivocally the hypothesis that $^1O_2$ is the mutagen we used a bound photosensitizer. Control experiments (data not presented) indicated that the dye is not eluted from the beads to the medium, cannot penetrate the cells and is recovered easily from the medium after incubation. No toxicity or mutagenicity can be demonstrated in the presence of the dye when either oxygen or light is omitted (Table 1). However, in the presence of all three

Table 1. The enhancement and inhibition of photodynamic mutagenicity in V79 cells.

Treatment	Plating efficiency (%)	Number of mutants per 10^6 cells plated	Number of mutants per 10^6 surviving cells
Control (full medium)[a]	94	0.3	0.3
Sen[a]	92	0.6	0.6
Sen (N_2 atmosphere)[b]	81	1.3	1.4
Sen[b]	29	4.0	12.8
0.1 mM β-carotene[b]	72	0.3	1.3
Sen + 0.1 mM β-carotene[b]	64	1.3	2.4
5mM DPBF[b]	80	1.3	1.6
Sen + 5mM DPBF[b]	38	2.6	6.5
Sen[b] + TPA (0.1 μg/ml)[c]	28	8.0	26.6
Sen[b] + TPA (0.5 μg/ml)[c]	24	10.0	39.1

The experimental conditions are described under Materials and Methods.
[a]These treatments were done in the dark.
[b]Cells were exposed to these compounds for 30 min. in the presence of light.
[c]TPA was added immediately after exposure and was present for the remainder of the experiment.

factors, (Sen, O_2 and light), a pronounced mutagenic effect is found which increased with exposure time (Fig. 1b). It is feasible to assume that a chemical species is produced outside the cell, and its formation is catalyzed by light and depends on the presence of Sen and oxygen. To support the hypothesis that $^1O_2$ which has a lifetime of only 2 μs in H_2O medium (11), causes the mutagenicity, we exchanged H_2O with D_2O. In this medium, the lifetime of $^1O_2$ is increased by a factor of 10 (11), and as expected, the toxicity and mutagenicity are impressively increased (Fig. 1b). β-carotene, a specific $^1O_2$ scavenger (12) and DPBF an $^1O_2$ trapper (13) inhibit the toxicity and mutagenicity effects. TPA, a potent cancer promoter has recently been shown to increase mutation frequency induced by mutagens in mammalian cells, while not being itself a mutagen (14). As shown in Table 1, TPA also enhanced $^1O_2$ mutagenicity in V79 cells.

A major question evolves from this study: Is $^1O_2$ a carcinogen? Recent studies in bacterial (15) and mammalian (16) systems have shown that most if not all carcinogens are mutagens. It was found that many carcinogenic polycyclic hydrocarbons show photodynamic activity (17). Early work showed that illumination of mice injected with solutions of photosensitive compounds produced skin tumors, whereas, neither illumination nor sensitizer alone gave this effect (18). After U.V. irradiation, Epstein (19) noted that skin tumors developed slower in β-carotene-treated mice compared to untreated controls. It has been suggested that accumulation of porphyrins in human skin with aging may result in photodynamic carcinogenesis (18). Bodaness and Chan (20) have recently suggested that skin cancer caused by U.V. might be mediated by $^1O_2$. These studies indicate that the role of singlet oxygen in carcinogenesis warrants further investigation.

REFERENCES

1. Boss, N., Saran, M., Lengfelder, E., Michel, C., Fuchs, F., and Frenzel, C. (1978) Photochem. Photobiol. 28, 629-638.
2. Foote, C.S. (1976) in Free Radicals in Biology, Pryor, N.A., Ed., pp. 85-133, Academic Press, New York.
3. Ito, T. (1978) Photochem. Photobiol. 28, 493-508.
4. Rahimtula, A.D., Hawco, F.J., and O'Brien, P.J. (1978) Photochem. Photobiol. 28, 811-815.
5. Smith, J.D., Freeman, G., Vogt, M., and Dulbecco, R. (1960) Virology 12, 185 196.
6. Spikes, J.D. (1977) in The Science of Photobiology, Smith, K.C., Ed., pp. 87-112, Plenum Press, New York and London.
7. Burki, H.J., and Lam, C.K. (1978) Mutation Res. 54, 373-377.
8. Jacobson, E.D., Krell, K., Dempsey, M.J., Lugo, M.H., Ellingson, O., and Hench, C.W. (1978) Mutation Res. 51, 61-75.
9. Imray, P.F., and Macphee, D.G. (1975) Mutation Res. 27, 299-306.
10. Gutter, B., Speck, W.T., and Rosenkranz, H.S. (1977) Mutation Res. 44, 177-182.
11. Merkel, P.B., and Kearns, D.R. (1972) J. Am. Chem. Soc. 94, 7244-7253.
12. Foote, C.S., and Denny, R.W. (1968) J. Am. Chem. Soc. 90, 6233-6235.
13. Matheson, I.B.C., and Lee, J. (1970) Chem. Phys. Lett. 7, 475-476.
14. Lancas, G.R., Baxter, C.S., and Christian, R.T. (1978) J. Toxicology and Environmental Health 4, 31-36.
15. McCann, J., Choi, E., Yamasaki, E., and Ames, B.N. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 5135-5139.
16. Hsie, A.W., Couch, B.D., San Sebastian, J.R., Sun W.N.C., Riddle, J.C., Brimes, P.A., Machanoff, R., Forber, N.L., Kenny, R.T., and Hsie, M.H. (1978) Proc. Am. Assoc. Cancer Res. 19, 93.
17. Malling, H.V., and Chu, E.H.Y. (1970) Cancer Res. 30, 1236-1240.

18. Santamaria, L., and Prino, G. (1964) in Research Progress in Organic, Biological and Medicinal Chemistry, Gallo, U. and Santamaria, L., Ed., pp. 260-336, Soueta Editoriale Farmacentica, Milan.
19. Epstein, J.H. (1977) Photochem. and Photobiol. 16, 117-119.
20. Bodaness, R.S., and Chan, P.C. (1979) Biochem. and Biophys. Res. Commun. 87, 1116-1123.