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Methylene Blue plus Light Mediates 8-Hydroxyguanine Formation in DNA

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Exposure to methylene blue (MB) plus light mediates formation of large levels of 8-hydroxyguanine in DNA. The amount of 8-hydroxy-2′-deoxyguanosine (8-OHdG) present in DNA increased as the amount of MB concentration increased throughout the 2 to 200 μM range studied and was dependent on light exposure. As the time of light exposure increased so did the 8-OHdG content to levels of about 750 8-OHdG/10^5 deoxyguanosine after 15 min of light exposure when MB was at 20 μM. Even though previous research has demonstrated that hydroxyl free radicals formed from a variety of sources mediate 8-OHdG formation in DNA, inclusion of mannitol, superoxide dismutase, catalase, and desferal in the MB plus light experiments demonstrated that these scavengers of oxygen free radical intermediates or precursors caused either no change or an increase in the 8-OHdG content of DNA exposed to MB plus light. These results appear to rule out the direct role of oxygen free radical intermediates in the primary events involved in the MB plus light mediated formation of 8-OHdG in DNA. Oxygen was essential to cause MB plus light mediated 8-OHdG formation in DNA. It was noted that when the reaction was carried out where the deuterium oxide content had been increased to 100%, the amount of 8-OHdG formed in DNA increased about threefold over that observed when comparable reactions were carried out in pure H_2O. Use of the singlet oxygen scavenger 2,5-dimethylfuran has yielded variable results on the MB plus light mediated formation of 8-OHdG in DNA. The data taken collectively clearly indicate that MB plus light mediates 8-OHdG formation in DNA. The D_2O data and the requirement for oxygen suggest that singlet oxygen may be an intermediate. © 1989 Academic Press, Inc.

Formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG)[2] either in DNA *or* from the free nucleoside deoxyguanosine (dG) has been shown to be mediated by hydroxyl free radicals (1–3) as well as by certain other reducing substances including hydrazines (1), polyphenols (4), and intermediates in carcinogen metabolism (5). A strong association between carcinogenesis and agents mediating formation of 8-OHdG in the DNA of target tissue has been demonstrated in Nishimura's laboratory (5–8). These largely indirect observations are supported by basic molecular studies demonstrating that miscoding of DNA readout occurs when 8-OHdG is preseht in the DNA template (9). The presence of 8-OHdG in nuclear DNA of normal tissue appears to be in the range of 1 to 58-OHdG/10^5 dG, yet it is present at much higher levels in mitochondrial DNA (10) and in chloroplast DNA (11). The imposition of a strong oxidative insult on a biological system has been shown to increase the level of 8-OHdG in DNA of the few systems which have been studied (5, 6, 11-13).

Methylene blue (MB) is a thiazine dye. The action of MB plus light on DNA has been investigated in a limited manner for over 20 years. Bellin and Oster (14) using

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[2] Abbreviations used: 8-OHdG, 8-hydroxy-2′-deoxyguanosine; dG, deoxyguanosine; MB. methylene blue.

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3.5 μM MB and a 500 W tungsten light at 15 cm demonstrated that inactivation of the transforming principle from *Diplococcus pneumoniae* occurred with a half-time of about 15 min. In a series of reports in the early 1960s, Simon and Van Vunakis (15-17) showed that MB plus light caused damage to guanine almost exclusively, when the nucleotides were treated either free in solution or as a part of DNA. The most effective wavelength of light was that which the dye absorbs maximally (16). The ability of poly(UG) to incorporate amino acids into peptides was found to be highly susceptible to MB plus light action and they found the guanine residue was selectively damaged (17). Waskell *et al.* (18) noted that extensive white light irradiation of guanosine in the presence of MB at alkaline pH caused formation of several breakdown products. Kornhauser *et al.* (19) showed that MB plus light reacted with guanosine in a manner such that different products were obtained with singlet oxygen generated by radiofrequency discharge as compared to MB plus light. Friedmann and Brown (20) utilizing polyoma I DNA demonstrated by sequencing techniques that MB plus light caused guanine-specific modifications and that piperidine treatment of the lesioned DNA caused chain cleavage at guanine residues.

Formation of 8-OHdG within the DNA structure independent of other extensive DNA lesions is a highly desirable research objective. Methods previously available using various sources of hydroxyl free radicals such as uv plus H_2O_2 (3) or by an ascorbate/iron reaction (21) produce either many lesions in addition to forming 8-OHdG in the former case or cause extensive strand breakage with low levels of 8-OHdG in the latter case. Thus with these restrictions in mind and in view of the previous observations showing specific reactivity of guanine, we treated DNA with MB plus light and have discovered that this treatment yields large levels of 8-OHdG within the treated DNA. These novel findings presented here clearly indicate that MB plus light mediates 8-OHdG formation in DNA. A preliminary report of this work has appeared previously (22).

MATERIALS AND METHODS

Chemicals and enzymes. Calf thymus DNA, DNAse I from bovine pancreas, endonuclease from *Neurospora crassa*, phosphodiesterase from *Crotalus durrius*, and alkaline phosphatase from calf intestine were all purchased from Calbiochem. MB from several sources has been used with similar results; however, most experiments were conducted with dye purchased from Sigma Chemical Co.

Quantitation of 8-OHdG and dG in DNA. The amount of 8-OHdG and the amount of dG present in the DNA digest was determined as described previously (2, 3, 12) by use of HPLC (IBM Instruments 9521 pump) with an optical detector (Perkin–Elmer LC15B uv detector with a *254* cutoff filter) in line with and before an electrochemical detector (IBM instruments EC/230 Amperometric detector). The dG content was quantitated by uv monitoring and 8-OHdG content was quantitated by electrochemical detection with the electrode potential set at 0.8 V (2, 3, 12). The mobile phase (pH 5.2) was 124 mM citrate, 25 mM sodium acetate, 30 mM NaOH, 10 mM citrate, and 4% methanol and the flow rate was 1 ml/min. The 8-OHdG standard was synthesized as described previously (2). DNA was digested to the nucleoside level as described previously (12).

Exposure of DNA to methylene blue plus light. Calf thymus DNA was solubilized to a concentration of 100 μg/ml in 0.014 M sodium phosphate buffer pH 6.8. MB was added to the DNA from a 2 mM stock solution (pH 5.0) in deionized water to various final concentrations between 2 and 200 μM. Care was taken in handling the samples to ensure only a low level of background light when MB and DNA were present together. DNA with MB present was exposed to white light from a 100 W tungsten lamp usually at a distance of 11 cm above a 100-ml beaker with a surface diameter of 4.5 cm. The DNA was placed in an ice bath and a 0.4-cm layer of H_2O in a petri plate was placed above the beaker to shield from infrared radiation (19). Immediately after light exposure NaCl was added to the DNA solution to make the final concentration 1.0 M and then 2.5 vol of ethanol was added to precipitate the DNA. The DNA was washed two times more with ethanol and then solubilized and digested to the nucleoside level (12). Ethanol precipitation removed MB from the DNA. In pilot experiments we found that MB when present during the enzymatic digestion procedure interfered with the action of the digestion enzymes. The amount of 8-OHdG present was quantitated as described previously (1.3.12).

Experiments designed to determine if oxygen was essential in the MB plus light experiments were conducted in an apparatus described previously where argon gas was deoxygenated using pyrogallol (21). The deoxygenated argon gas was bubbled through 10 mM Tris buffer, pH 8.0, containing 1 mM EDTA and 0.1 mM diethylenetriaminepentaacetic acid contain-

TABLE I

METHYLENE BLUE PLUS LIGHT[a] INDUCED 8-OHdG FORMATION IN DNA

Treatment	dG Content	dG/T	8-OHdG/10^5 dG
No MB	159 ± 19.8 (4)	0.95	0[b]
2 μM MB	159 ± 21.0 (4)	0.93	200.0 ± 21.0 (4)
20 μM MB	155 ± 9.2 (4)	0.89	792.5 ± 90.2 (4)
200 μM MB	148 ± 15.3 (4)	0.85	1983.3 ± 348.2 (4)

[a] The light duration was 15 min.

[b] The 8-OHdG content was not detectable at the sensitivity settings employed; the usual level is from 3 to 12 8-OHdG/10^5 dG depending upon the particular batch of DNA used.

ing 100 μg/ml DNA and MB (200 μM) for 2 h in darkness. A comparable sample was exposed to air during exposure to light. Light from a 250 W incandescent light (Sylvania 401 superflood BBA), 20 cm from the DNA solution, was passed through a water filter to absorb heat. Samples were removed and immediately added to ethanol for MB removal and DNA precipitation at various times after turning on the light, using ports and pipets filled with argon such as not to introduce oxygen into the argon samples. The precipitated DNA samples were processed for 8-OHdG analysis as described above.

RESULTS

MB plus light mediates formation of large levels of 8-OHdG within the DNA as the results in Table 1 demonstrate. No increase in 8-OHdG content above background levels occurs when MB-treated DNA remains in the dark. When the MB level was 200 μM the amount of 8-OHdG formed within DNA was nearly equivalent to 2% of the dG present. The amount of 8-OHdG formed within MB-treated DNA is dependent on the length of time of light exposure as the results in Fig. 1 demonstrate. The increase was essentially linear with time for the first 15 min but then became less per unit time as the light exposure continued.

We have tested the effect of various oxygen radical scavengers and protective enzymes on MB plus light induced 8-OHdG formation in DNA. The results are presented in Tables II and III. Results shown in Table II demonstrate that superoxide dismutase and/or catalase had very little, if any effect. Superoxide dismutase has been shown to be very resistant to the action of MB plus light (23). Mannitol, which scavenges hydroxyl free radicals, did not cause any significant change in the amount of 8-OHdG formed in DNA by MB plus light as the results in Table III demonstrate. Desferal, which chelates iron, also did not cause a decrease in the amount of 8-OHdG formed in MB plus light treated DNA (data not shown).

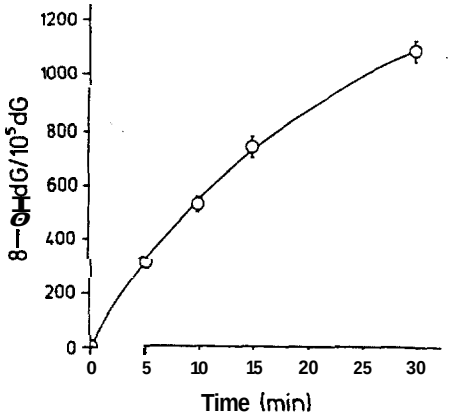

FIG. 1. Increase in 8-OHdG content of DNA treated with methylene blue and exposed to white light for various periods of time. The methylene blue concentration was 20 μM and the data points represent the means of five individually treated samples analyzed at each time point. The standard error of the mean is delineated by error bars at each point.

TABLE II

EFFECT OF SUPEROXIDE DISMUTASE AND CATALASE ON METHYLENE BLUE PLUS LIGHT MEDIATED 8-OHdG FORMATION IN DNA[a]

Treatment	8-OHdG/10^5 dG
MB plus light only	596 ± 58.9 (4)
MB plus light + SOD[b]	612 ± 102.0 (4)
MB plus light + catalase[b]	822 ± 23.2 (4)
MB plus light + SOD + catalase	772 ± 37.6 (4)

[a] Calf thymus DNA, 100 μg, in 2 ml of 14 mM Na phosphate buffer, pH 6.8; MB was present at 20 μM and light exposure (15 min) was as in Table I.

[b] SOD and catalase were present at 200 μg/ml.

TABLE III

EFFECT OF MANNITOL ON METHYLENE BLUE PLUS LIGHT MEDIATED 8-OHdG FORMATION IN DNA[a]

Treatment	8-OHdG/10^5 dG
No MB, light, mannitol	4.2 ± 5.2 (4)
MB, light, no mannitol	727.5 ± 25.8 (4)
MB, light, mannitol	750.3 ± 37.7 (4)

[a] DNA was exposed to light with or without methylene blue (20 μM) or mannitol (100 mM) present in a procedure as outlined in Tables I or II.

Data presented in Fig. 2 clearly demonstrate a requirement for oxygen in the MB plus light mediated formation of 8-OHdG in DNA. The results presented in Fig. 3 show that as the D_2O content is increased, there is an increase in the amount of 8-OHdG formed in DNA by MB plus light and, in fact, at 100% D_2O, the amount formed is threefold higher than the amount formed at 100% H_2O. It is likely that in 100% D_2O 8-ODdG is formed rather than 8-OHdG. We are conducting experiments to resolve this question. We have examined the effect of 2,5-dimethylfuran, a good singlet oxygen scavenger on MB plus light mediated formation of 8-OHdG in DNA. The results obtained have been variable and appear to be dependent upon a number of factors including the presence or absence of ethanol as well as the ionic strength of the buffer.

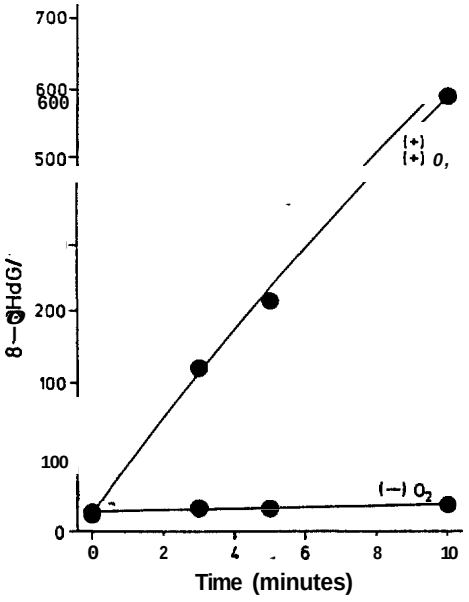

FIG. 2. Increase in 8-OHdG content of DNA treated with methylene blue plus light in the presence or absence of oxygen.

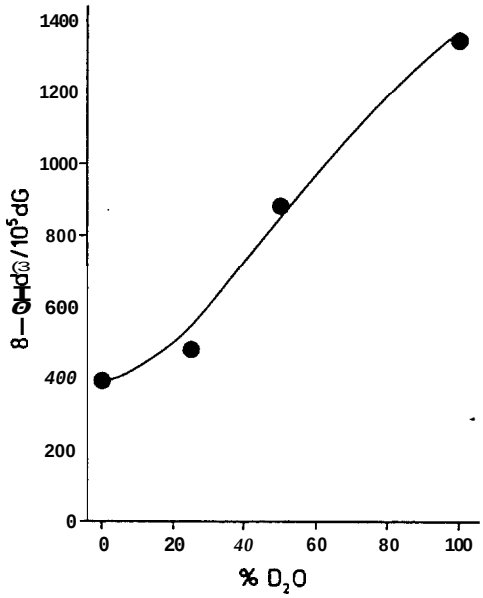

FIG. 3. Increase in 8-OHdG content of DNA treated with methylene blue plus light with increasing concentration of D_2O.

DISCUSSION

The biological significance of the presence of 8-OHdG within DNA is a subject of intense research interest at the present time. Nishimura's research group has presented compelling evidence that 8-OHdG in DNA is associated with conditions of enhanced carcinogenesis (5–8). Richter *et al.* (10) recently demonstrated that the 8-OHdG content of mitochondrial DNA of normal rat liver is significantly higher than that of nuclear DNA and has speculated that the enhanced genomic divergence of mitochondrial DNA with time can

possibly be explained by the increased 8-OHdG content. The theoretical basis for the mutagenic potential of 8-OHdG is based on the demonstration of Kuchino *et al.* (9) that *in vitro* incorporation of non-complementary nucleotides. by DNA polymerase I of *Escherichia coli* occurs not only opposite 8-OHdG but also opposite residues adjacent to 8-OHdG in the DNA template.

Understanding the role of 8-OHdG in the etiology of normal and pathological conditions has been hampered by the difficultyin forming 8-OHdG in DNA independent of other lesions in the DNA. It has been clearly demonstrated that hydroxyl free radicals will not only form 8-OHdG (1–3), but also cause strand breakage (21), formation of thymine glycol (24), 5-hydroxymethyl uracil (25), and most likely many other lesions in DNA. The older literature demonstrating that MB plus light caused damage in DNA specifically at guanine (15–17) prompted us to determine if 8-OHdG may be formed by this treatment. The present report represents the first demonstration of this fact and as such appears to be a significant development in the long-range goal of understanding the role of 8-OHdG in normal and pathological processes.- Experiments we have conducted (and not presented) with the four DNA nucleosides free in solution have demonstrated that MB plus light causes loss in dG only with concomitant formation of 8-OHdG. In addition monitoring thymidine change versus dG changes in DNA which has been treated with MB plus light versus no treatment shows that dG is selectively lost in relation to thymidine (Table 1), as had been reported much earlier by Simon and Van Vunakis (16).

The mechanism by which MB plus light uniquely causes 8-OHdG formation in DNA is not known, but the data presented certainly appear to establish a mechanism independent of oxygen free radical intermediates. Regarding mechanisms of 8-OHdG formation independent of hydroxyl free radicals, Kohda *et al.* (5) postulated that the N-acyl ester of 4-hydroxyaminoquinoline 1-oxide formed the nitrinum ion which then added to the N-7 of guanine resulting in water addition to the C-8 of guanine which then breaks down to yield 8-OH guanine. Cadet *et al.* (26) observed enhanced 8-OHdG formation during ionizing irradiation if the radiosensitizer TAN was present. They postulated a radical cation intermediate of guanine to which water added eventually yielding 8-OHdG.

In general most photosensitizers absorb light forming a transitory excited singlet state molecule which then relaxes to a longer lived excited triplet state molecule which is the intermediate that initiates most photochemical reactions (26). MB appears to act through an excited triplet state as a photosensitizer (27). In general, the excited triplet state photosensitizer reacts either by Type I mechanisms, whereby the triplet state sensitizer interacts with another molecule to cause an electron or hydrogen atom transfer, or by Type II mechanisms, where singlet oxygen is formed (27). Therefore, the following reactions most likely apply to MB plus light induced damage to DNA, where MB^{*1} and MB^{*3} refer to the excited single and triplet states of MB, respectively:

$$MB \xrightarrow{h\nu} MB^{*1}(\text{singlet}) \rightarrow MB^{*3}(\text{triplet}) \quad [1]$$

$$MB^{*3} \rightarrow \text{H or e transfer} \quad \text{(Type I)} \quad [2]$$

$$MB^{*3} \xrightarrow[\text{Fast}]{O_2} {}^1O_2 + MB \quad \text{(Type II)} \quad [3]$$

$$MB^{*3} \xrightarrow[\text{Slow}]{O_2} O_2^- + MB^+ \quad \text{(Type II)} \quad [4]$$

Our data indicate that possibly singlet oxygen is an essential intermediate in the MB plus light mediated formation of 8-OHdG in DNA. The pertinent data can be summarized as such: (A) inhibitors or scavengers of oxygen free radicals do not inhibit 8-OHdG formation; (B) oxygen is essential for the reaction to occur; (C) when the reaction is carried out in D_2O, there is a threefold increase in 8-OHdG yield. It is known that the lifetime of singlet oxygen in D_2O is nearly 20 times longer than that of H_2O (27). Since the experiments with 2,5-dimethyfuran have yielded variable results, we are not certain if singlet oxygen is an essential intermedi-

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ate in the reaction. Even though 2,5-dimethylfuran is an excellent scavenger of singlet oxygen produced by MB plus light (28), it appears that MB exhibits specific binding domains in DNA depending upon ionic strength as well as other parameters (29). Therefore, the exact intermediate steps in MB plus light mediated 8-OHdG in DNA require further investigation.

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REFERENCES

1. KASAI, H., AND NISHIMURA, S. (1984) *Nucleic Acids Res.* 12, 2137–2145.
2. FLOYD, R. A., WATSON, J. J., WONG, P. K., ALTMILLER, D. H., AND RICKARD, R. C. (1986) *Free Radical Res Commun.* 1, 163–172.
3. FLOYD, R. A., WEST, M. S., ENEFF, K. L., HOGSETT, W. E., AND TINGEY, D. T. (1988) *Arch Biochem. Biophys.* 262, 266–272.
4. KASAI, H., AND NIHSIMURA, S. (1984) *Gann* 75. 565–566.
5. KOHDA, K., TADA, M., KASI, H., NISHIMURA, S., AND KAWAZOE, Y. (1966) *Biochem Biophys. Res Commun.* 139, 626–632.
6. KASAI, H., NISHIMURA, S., KUROKAWA, Y., AND HAYASHI, Y. (1987) *Carcinogenesis* 8, 1959–1961.
7. KASAI, H., AND NISHIMURA, S. (1984) *Gann* 75. 841–844.
8. KASAI, H., TANOOKA, H., AND NISHIMURA, S. *Gann* 75,1037–1039.
9. KUCHINO, Y., MORI, F., KASAI, H., INOUE, H., IWAI, S., MIURA, K., OHTSUKA, E., AND NISHIMURA, S. (1987) *Nature (London)* 327, 77–79.
10. RICHTER, C., PARK, J.-W., AND AMES, B. N. (1988) *Proc. Natl. Acad. Sci. USA* 85,6465–6467.
11. FLOYD, R. A., WEST, M. S., HOGSETT, W. E., AND TINGEY, D. T. (1989) *Plant Physiol.*, in press.
12. FLOYD, R. A., WATSON, J. J., HARRIS, J., WEST, M., AND WONG, P. K. (1986) *Biochem. Biophys. Res. Commun.* 137, 841–848.
13. KASAI, H., CRAIN, P. F., KUCHINO, Y., NISHIMURA, S., OOTSUYAMA, A., AND TANOOKA, H. (1986) *Carcinogenesis* 7, 1849–1851.
14. BELLIN, J. S., AND OSTER, G. (1960) *Biochim. Biophys. Acta* 42, 533–535.
15. SIMON, M. I., AND VAN VUNAKIS, H. (1962) *J. Mol. Biol* 4,488–499.
16. SIMON, M. I. AND VAN VUNAKIS, H. (1964) *Arch. Biocha Biophys.* 105,197–206.
17. SIMON, M. I., GROSSMAN, L., AND VAN VUNAKIS, H. (1965) *J. Mol. Biol* 12.50–59.
18. WASKELL, L. A., SASTRY, K. S., AND GORDON, M. P. (1966) *Biochim. Biophys. Acta* 129, 49–53.
19. KORNHAUSER. A., KRINSKY, N. I., HUANG, P.-K.C., AND CLAGETT, D. C. (1973) *Photochem. Photobiol.* 18, 63–69.
20. FRIEDMANN, T. AND BROWN, D. M. (1978) *Nucleic Acids Res* 5,615–622.
21. SCHNEIDER, J. E., BROWNING, M. M., AND FLOYD, R. A. (1988) *Free Radical Biol Med.* 5,287–295.
22. FLOYD, R. A., WEST, M. S., ENEFF, K., SCHNEIDER, J. E., AND ZHU, X. (1989) *Toxicologist* 9, 13. [Abstract 51]
23. FORMAN, H. J., EVANS, H. J., HILL, R. L., AND FRIDOVICH, I. (1973) *Biochemistry* 12, 823–827.
24. TEEBOR, G. W., BOORSTEIN, R. J., AND CADET, J. (1988) *Int. J. Radiat. Biol.* 54, 131–150.
25. TEEBOR, G. W., FRENKEL, K. AND GOLDSTEIN, M. S. (1984) *Proc. Natl. Acad Sci. USA* 81, 318–321.
26. CADET, J., BERCER, M., AND SHAW, A. (1986) *in* Mechanisms of DNA Damage and Repair (Simic, M. G., Grossman. L. and Upton, A. D., Eds.), pp. 69–74. Plenum, New York.
27. FOOTE, C. S. (1976) *in* Free Radicals In Biology (Proyer, W. A. Ed.), Vol. 2, pp. 85–133, Academic Press, New York.
28. USUI, Y., AND KAMOGAWA, K. (1974) *Photochem. Photobiol.* 19, 245–247.
29. VAN DER PUTTEN, W., AND KELLEY, J. M. (1989) *Photochem. Photobiol.* 49, 145–151.