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The Combined Action of Chemical Carcinogens on DNA Repair in Human Cells*

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Summary. Excision repair was studied in normal human and ataxia telangiectasia (AT) cells proficient in repair of UV and its mimetic chemicals, and in xeroderma pigmentosum group C (XP C) cells (deficient in repair of UV and its mimetics), after treatment with several combinations of chemical carcinogens, by the photolysis of bromodeoxyuridine incorporated into parental DNA during repair. Results indicate that repair was additive in AT, and XP C cells treated with N-acetoxy-2-acetylaminofluorene (AAAF) plus ethyl methanesulfonate (EMS) or methyl methanesulfonate (MMS) indicating that there are different rate limiting steps for removal of both types of damage. Data on the combinations of 4-nitroquinoline 1-oxide (4NQO) plus MMS or EMS are difficult to interpret, but they do not indicate inhibition of DNA repair.

Introduction

Previous studies on DNA excision repair in human cells treated with repair saturating doses of ultraviolet (UV) and mimetic carcinogens showed that in repair proficient cells the total repair was additive for UV and N-acetoxy-2-acetylaminofluorene (AAAF) [1, 2] or for UV and doses of ICR-170 and 4-nitroquinoline 1-oxide (4NQO) low enough to inhibit unscheduled DNA synthesis (UDS) [4], implying that there are different rate limiting steps in removal of both the physical and the chemical damages. The results were more complicated for xeroderma pigmentosum (XP) cells in that UV plus AAAF or UV plus ICR-170 showed appreciably less repair than after UV alone, whereas UV plus 4NQO showed an additive effect [2, 4]. These results implied that for the UV mimetics, AAAF and ICR-170, repair deficient XP cells have a different repair system, not just fewer repair enzymes than normal cells; and that the same long patch repair system works on 4NQO damage in both normal and XP cells. In the present work the

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extent of repair after treatment with various combinations of the above UV-mimetic carcinogens and the X-ray mimetics [alkylating agents ethyl methanesulfonate (EMS) and methyl methanesulfonate (MMS)] was investigated in UV-excision repair proficient human cells and in UV-excision repair deficient XP C cells.

The technique of photolysis of 5 bromo-2′-deoxyuridine (BrdUrd) [14] incorporated into parental DNA during repair was used to study excision repair after the various treatments. The technique is sensitive, gives repeatable results and agrees well with other techniques used for measuring repair such as UDS and assays for UV-endonuclease sensitive sites [2, 3]. The results indicate that in AT and XP C cells repair is additive after a combined action of AAAF plus EMS or MMS. The data for 4NQO plus MMS or EMS are not easy to interpret because in these chemicals no saturation of the excision repair system seems to have been achieved.

Materials and Methods

Cell Culture. Normal human fibroblasts, Rid Mor (CRL 1220) and ataxia telangiectasia Ne No (CRL 1347) (proficient in repair replication after γ-irradiation [12]; and xeroderma pigmentosum (XP) group C, Ge Ar (CRL 1161) from the American Type Culture Collection (Rockville, MD) were used. Cells were grown in plastic dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 400 μg/ml L-glutamine, 140 units/ml penicillin and 140 μg/ml streptomycin (Grand Island Biological Co., Grand Island, NY) and kept in a humidified 7.5% CO_2 atmosphere at 37° C. When cells reached confluency they were subcultured at a ratio of 1 : 3. Passages between 3 and 24 were used.

Cells were regularly checked for the absence of mycoplasma contamination by fluorescent staining with bisbenzimidazol compound 33258 (Hoechst Pharmaceutical Co., Kansas City, MO) as described by Chen [6].

Chemical Treatment. A stock solution of 5 mM AAAF (NCI Standard Chemical Carcinogen Reference Repository) was dissolved in fresh $(CH_3)_2SO$ and stored in plastic vials under liquid nitrogen. AAAF was added to cells in culture medium containing serum to a final concentration of 20 μM. After 30 min at 37° C the medium was changed.

A stock solution of 10 mM 4NQO (ICN Pharmaceutical, Plainview, NY) in Hank's balanced salt solution (Grand Island Biological Co.) was prepared and kept frozen at −10° C. Working solutions of 0.01−1 mM were prepared and added to cells in petri dishes to the required final concentration in culture medium for 30 min before medium change.

Ethyl methanesulfonate and methyl methanesulfonate were obtained from Eastman Kodak Co. (Rochester, NY). Immediately before use they were diluted in fresh $(CH_3)_2SO$ to 500 mM. From this stock, cells were treated in the medium with the required final concentration for 30 min before the medium was changed.

Bromodeoxyuridine Photolysis. Details of this technique and its advantages in studying repair synthesis were given previously [13−15]. Two plates of cells per assay, each labeled with a different radioactive precursor, were used. Approximately 100,000 cells were plated in 5 ml of medium in 60-mm plastic dishes. One plate was labeled for 36 h with [^{3}H]-deoxythymidine (dThd) (0.4 μCi/ml, 6.7 Ci/m mol; New England Nuclear, Boston, MA), and the second plate was labeled for 36 h with [^{14}C]-dThd (0.4 μCi/ml, 50 Ci/mol; New England Nuclear). After the labeling period, the medium was discarded, and the cells in both plates were exposed to chemical treatment (single or combined). Immediately after treatment, cells were incubated in fresh medium containing 4 mM hydroxyurea (in other instances no hydroxyurea or 2 mM hydroxyurea were used without change in the results) and 0.1 mM BrdUrd (Sigma Chemical Co., St. Louis, MO) for cells labeled with [^{3}H]-dThd or containing hydroxyurea and 0.1 mM dThd (Sigma) for cells labeled with [^{14}H]-dThd. Ahmed and Setlow [4] have shown that 4 mM hydroxyurea had no effect on UDS, nor did it affect removal of endonuclease sensitive sites from UV-irradiated cells. Moreover, cell tox-

icity studies employing the dye trypan blue 24 h after treatment with 4 mM hydroxyurea [4] showed that the cells excluded the dye indicating that they were alive.

Both plates of cells were incubated at 37° for 24 h to allow repair and incorporation of BrdUrd or dThd without any deleterious effects observed on cells. They were then washed with phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 2.0 g $Na_2H\ PO_4$, and 0.4 g KH_2PO_4 in 1 liter distilled H_2O, pH 7.2) and exposed to 2000 R X-ray to facilitate strand separation in the subsequent alkaline sedimentation [9]. The cells were harvested and mixed together at 10^6 cells/ml in cold EDTA: 0.8% NaCl solution [16]. The cells in solution (0.2 ml) in a quartz microcuvette were exposed up to 6 min to 313 nm radiation from a Johns monochromator [11] (courtsies of J. C. Sutherland and R. B. Setlow, Biology Department, Brookhaven National Laboratory). Any scattered radiation less than 305 nm was absorbed by a thin film of mylar [10].

Fifty thous and cells were lysed on top of an alkaline sucrose gradient (5 to 20%, 2 M NaCl) and the DNA was centrifuged for convenience at 30,000 rpm for 130 min in a SW 50.1 rotor [16] in polyallomer tubes in a Beckman model L-5 centrifuge. Drops were collected from a hole punched in the bottom of each tube onto strips of filter paper, and the acid-insoluble radioactivity in ^3H and ^{14}C was measured in a toluene-based scintillation fluid in a Packard scintillation counter. The distributions of radioactivity were converted to weight-average molecular weights by a computer program based on the distances sedimented by phage DNA's of known molecular weights: T_4 DNA, 60×10^6; λ DNA, 15×10^6; T_7 DNA, 13×10^6 and ϕX 174 DNA, 1.7×10^6 as described previously by Regan et al. [14] and by Regan and Setlow [13]. The amount of radioactivity per gradient was between 4000 and 3000 cpm for each isotope. Because in calculation of molecular weights I started at four fractions from the top of the gradient. M_w of cells incubated in BrdUrd or dThd was used for different 313 nm doses rather than M_n since the latter is very sensitive to fluctuations in the amount of radioactivity near the top of the gradient [2]. The difference between their reciprocals $\Delta\ (1/M_w) = (1/M_w)_{BrdUrd} - (1/M_w)_{dThd}$ is a measure of the photolysis of BrdUrd incorporated into parental DNA during repair, hence, of repair itself.

The doses of 313 nm are presented in terms of the number of breaks per BrdUrd residue that would be made by exposure to the radiation source. In actual experiments the dose was measured with a calibrated photocell whose output multiplied by time could be expressed in breaks per BrdUrd. A typical dose of 5.1×10^4 J/m^2 corresponds to 4.2×10^{-2} breaks/BrdUrd.

Results

Figure 1 shows representative alkaline sedimentation profiles of cells treated with various combinations of chemicals. In Fig. 1a, f dashed lines represent cells incubated in either BrdUrd or dThd without exposure to 313 nm photolysis. Such profiles were typical of all experiments. Values of $\Delta(1/M_w)$ for untreated cells were nearly zero.

Note that treated cells incubated in BrdUrd are photolyzed more extensively than treated cells incubated in dThd. Such differences are usually not observed in the absence of photolysis (see below). The dose of AAAF used (20 μM) was previously shown to saturate repair [2]. 4 NQO gave the highest repair level at 5 μM followed by a rapid decline in both cases (data not shown). Therefore, the dose employed for this chemical did not exceed 5 μM. The alkylating agents gave the maximum level of repair at 10 mM (Fig. 2). A dose of 0.1 M of these agents was tried. At this concentration, M_w of dThd DNA without 313 nm photolysis was $\sim 50 \times 10^6$. The highest photolytic dose caused extensive fragmentation of DNA, and it sedimented near the top of the gradient. Such gradients were difficult to analyze. There did not, however, seem to be appreciable inhibition of DNA repair at this concentration (0.1 M), though it is difficult to judge whether or not saturation of repair was achieved at the 10 mM concentration. Notice that the DNA of the different cell strains used was sensitized differently after treatment

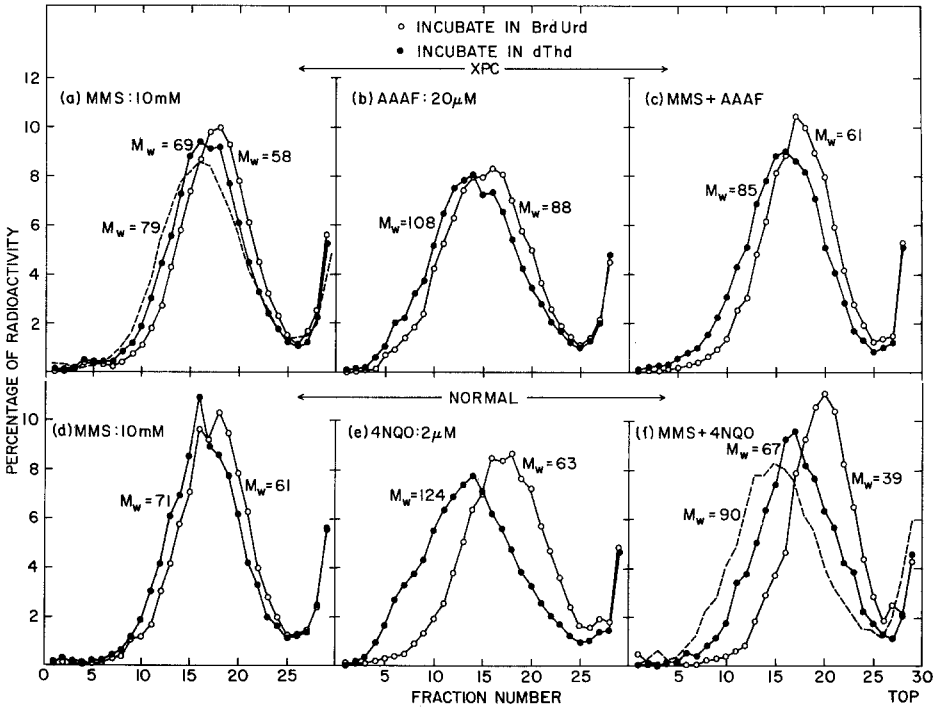

Fig. 1. Alkaline sucrose gradient profiles of DNA from XP C cells (a–c), and normal human cells (d–f). Cells were treated with the chemicals as indicated and then incubated for 24 h in non-radioactive BrdUrd or dThd and exposed to 313 nm photolytic dose of 4.2×10^{-2} breaks/BrdUrd. M_w in millions are indicated on the profiles. In a, e and f dashed lines represent profiles of dThd DNA without 313 nm photolysis. Sedimentation to the left

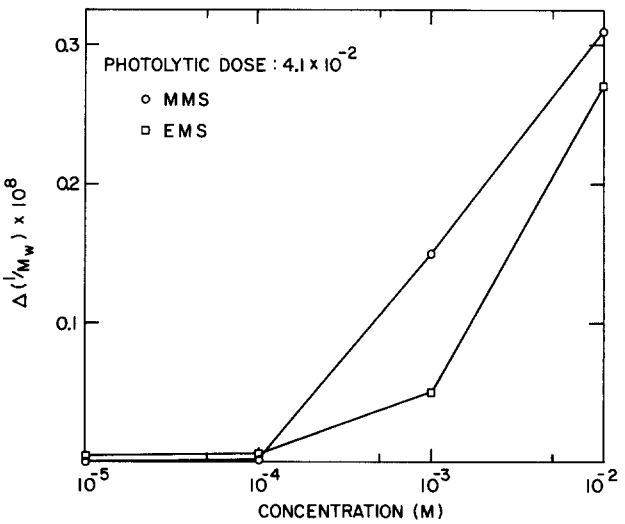

Fig. 2. The relationship between $\Delta(1/M_w)$ as a result of photolysis by 313 nm and concentration of EMS and MMS observed in AT cells. Cells were treated with the chemicals as indicated and then incubated for 24 h in non radioactive BrdUrd or dThd and exposed to 313 nm photolytic dose of 4.1×10^{-2} breaks/BrdUrd

Table 1. Repair[a] in 24 h determined by BrdUrd photolysis in human cells after various treatments

Treatment	$\Delta(1/M_w) \times 10^8$		
	Normal	AT	XP C
20 μM AAAF	1.4	1.6	0.21
2 μM 4NQO	0.78	0.86	0.51
10 mM EMS	0.22	0.22	0.26
10 mM MMS	0.23	0.33	0.27
10 mM MMS + 20 μM AAAF	1.7	2.0	0.46
+ 2 μM 4NQO	1.1	1.8	0.73
10 mM EMS + 20 μM AAAF	2.0	1.9	
+ 2 μM 4NQO		1.0	

[a] Repair determined as $\Delta(1/M_w) \times 10^8$ at photolytic dose 4.2×10^{-2} breaks/BrdUrd

with the alkylating agents, but the absolute value of repair was nearly the same (Fig. 1a and d). It is noticed that in Fig. 1a–c the M_w of dThd varies from 69 to 108 to 85, whereas the M_w of the BrdUrd strand varies from 58 to 88 to 61. Since both DNAs were run together in the same gradient, then these M_w values are functions of handling, but the $\Delta(1/M_w)$ values are consistent.

XP C cells were defective in repair of all UV-mimetic chemicals tested as in the case of UV [2, 4]. The extent of defect varied from 15–65% of repair proficient cells (Table 1). On the other hand, XP C cells were not different from normal cells in the repair of damage after treatment with alkylating agents. AT (Ne No) cells were previously reported to be as proficient as normal human cells in repair of UV and AAAF lesions [1], and were found in this study to repair damage from alkylating agents in a manner similar to normal and XP C cells. A summary of the data for the various combinations are presented in Table 1. Because in XP C cells the extent of repair was similar after a repair-saturating dose of AAAF and a dose of MMS that approximately gave the maximum level of repair, additivity of repair was readily observed (Table 1). In AT cells additivity of repair was also noticed in cells treated with AAAF and EMS. In normal human, AT and XP C cells treated with combinations of MMS plus 4NQO, repair was more than each treatment separately as in the case of AT cells treated with 4NQO plus EMS.

Discussion

Previous studies showed that high concentrations of 4NQO resulted in a rapid decline of UDS in human [4, 17] and hamster [18] cells, and high doses of MMS led to decrease in repair replication in human cells [8]. The AT cell strain used (Ne No) (CRL 1347) was previously shown [12] to be as proficient as normal human cells in excision repair after γ-irradiation. The present study shows it to be as proficient as normal human and XP C cells in removal of damage caused by alkylating agents. In the present study a

concentration of AAAF that resulted in saturation of repair [1, 2] was used; and 4NQO, EMS and MMS concentrations that did not lead to a decrease in repair were employed. Different DNA damaging chemicals induce different lesions in the DNA, but they can generally be grouped into two categories: 1) those that result in UV or "long" type repair such as AAAF and 4NQO, and 2) those that result in ionizing or "short" type repair such as EMS and MMS [13].

In the design of these experiments a concentration of a chemical that saturated repair (AAAF), and concentrations of 4NQO, EMS and MMS that did not lead to inhibition of repair were employed. In case of combinations of AAAF plus any of the other chemicals one can interpret the data if repair is additive, the same or inhibitory. On the other hand, in case of cells treated with combinations of any two chemicals that do not show saturation of repair the data become difficult to analyze because: 1) if inhibition of repair is observed this can be attributed to the toxic effect exerted by the high concentrations of the chemicals on the cells, and 2) additivity of repair would not mean separate pathways for repair of damages.

Previous reports have shown different kinetics of UDS in human lymphocytes treated with UV, NH_2, γ-rays and MMS indicating different rate limiting steps for removal of the various damages; though a common mechanism was implicated in the case of UV and NH_2 damages or MMS and γ-ray-induced damages [7]. Present results show that the extent of excision of damage from UV-mimetic chemicals in XP C cells was 15–65% that of normal or AT cells, but that they were as proficient as normal and AT cells in removal of damage caused by alkylating agents. This seems consistent with previous findings by Cleaver [8] who showed that XP cells were defective in excision repair of UV radiation but gave the same extent of repair replication compared to normal human cells after treatment with alkylating agents.

Brown et al. [5] reported that repair after UV plus 4NQO or AAAF was much less than the sum of the repair due to each agent separately, and that no additivity was observed after treatment with 4NQO and AAAF. In their paper they discussed earlier work by Ahmed and Setlow [1, 2] on UV plus AAAF and suggested that the contradictions between their data and Ahmed and Setlow's data could be due to: 1) level of UV doses used, and 2) the medium in which reactions were run. They stated that Ahmed and Setlow's dosimetry with UV-endonuclease from *Micrococcus luteus* indicated a yield of 1.8 sites/10^7 daltons at 10 J/m^2 in contrast to their 2.5 sites. This statement is not accurate since Ahmed and Setlow have shown that at 20 J/m^2 of UV there were 5.16×10^{-7} endonuclease breaks/dalton [2]. Moreover, Ahmed and Setlow have shown that repair saturated at a dose of 20 J/m^2 and remained constant up to a dose of 80 J/m^2 [3]. Regarding the second point, Brown et al. treated their cells with chemicals (especially AAAF) in serum free medium since the chemical is highly reactive, whereas Ahmed and Setlow treated their cells in a medium containing 10% fetal calf serum. However, in a repeat experiment performed in serum free medium, I found that AAAF did not lead to inhibition of removal of UV-endonuclease sensitive sites. Because no pharmacokinetic studies were reported by both independent investigators, no definitive correlation between the effect of culture factors and drug dose can be made at this time [19]. Ahmed and Setlow showed both experimentally and mathematically that the three techniques they used to measure excision repair (UDS, BrdUrd photolysis, and endonuclease sensitive sites) gave the same results [2]. Moreover, my previous experimental observations showed that the BrdUrd photolysis technique is a reproducible

and a highly informative technique; it allows estimation of both number and size of the repaired regions. That is why it was the method of choice. I do not know why these differences exist between the work of Ahmed and Setlow [1–4] and Brown et al. [5]; but they state that repair of 4NQO damage, determined by the repair replication technique, saturated at ~ 4 μM. I do not believe that in this situation this technique is accurate enough since Ahmed and Setlow [4] using UDS and BrdUrd photolysis technique, and others [17, 18] using UDS have shown that there is no saturation of the repair system after treatment with 4NQO.

The results on combinations of AAAF plus EMS or MMS show additivity of repair. Because a saturating dose of AAAF was used, additivity indicates that there are different rate limiting steps for removal of both types of lesions. On the other hand, the data on combinations of 4NQO plus EMS or MMS, are difficult to interpret because no saturation of the repair systems seem to have been achieved for these chemicals; but the data, however, do not indicate inhibition of DNA repair.

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