KINETICS OF DNA REPAIR IN ULTRAVIOLET-IRRADIATED AND *N*-ACETOXY-2-ACETYLAMINOFLUORENE-TREATED MAMMALIAN CELLS

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ABSTRACT Repair kinetics after saturating doses of ultraviolet radiation (UV), *N*-acetoxy-2-acetylaminofluorene (AAAF), and combinations of both agents were studied in human fibroblasts proficient and deficient in excision repair, and in Chinese hamster cells (V-79) deficient in excision repair. Three techniques were used: unscheduled DNA synthesis, photolysis of DNA repaired in the presence of bromodeoxyuridine (BrdUrd), and measurements of sites sensitive to a UV-endonuclease. The repair rate appears to be approximately constant during the first few hours after treatment. Later there is a decrease with time; the magnitude of the decrease depends on the cell line. Our data show that the decrease in repair observed in repair-deficient cells treated with combinations of both agents as compared to separate treatments is due neither to the cytotoxic effects of the agents used, nor to a shutoff of the repair system by the high concentrations of AAAF employed.

INTRODUCTION

Many mammalian cells may repair damage to their DNA resulting from ionizing and nonionizing radiation and from a variety of chemicals by excision repair mechanisms involving several enzymatic processes (1). In the case of ultraviolet radiation (UV) and the carcinogen *N*-acetoxy-2-acetylaminofluorene (AAAF) the amount of repair between 3 and 24 h saturates at about 20 Jm^{-2} of UV and 20 μM AAAF (2–5). Several techniques employed in measuring repair have shown equivalence between UV and AAAF doses (5–8), but saturation of repair at high doses might be due to saturation of either damage or repair. For UV the kinetics of pyrimidine dimer formation are the same in vivo and in vitro (9). Dimer formation is proportional to dose (3, 10) and does not saturate until above 100 Jm^{-2} of 254 nm (11). For AAAF less extensive data also show that damage is proportional to dose at low doses (12), although damage might saturate at high dose. Hence, it is the repair systems that seem to saturate at doses $\geq 20\ Jm^{-2}$ of 254 nm or $\geq 20\ \mu$M AAAF.

In many ways the excision repair of AAAF damage in mammalian cells mimics the repair of UV damage. Both are repaired by a "long patch" scheme (8), and cells deficient in the repair of UV damage are equally deficient in the repair of AAAF damage (8, 13, 14). UV-sensitive cells are also more sensitive to the lethal and muta-

genic effects of AAAF (15). Nevertheless, when cells proficient in excision repair were treated with UV and with AAAF at doses that individually gave saturating repair levels, the total repair was additive (6, 16). However, in cells deficient in excision repair, the total repair after a combined treatment was much less than additive and often less than that of individual treatments (16, 17). We were concerned that these surprising (to us) findings might result from some strange kinetics of repair—for example, AAAF damage to DNA of repair-deficient cells might be repaired only for a short time after treatment—because of some effects of AAAF on the repair system itself. Such a hypothetical shutoff could greatly decrease the amount of repair of UV damage. Hence, we compared the kinetics of repair of UV and AAAF damage.

Several investigators had reported kinetic studies on UV and AAAF repair in mammalian cells (5, 18–22), but most of these studies were done at doses below those required to achieve saturation and did not compare repair kinetics in repair-proficient and repair-deficient mammalian cells. To fill in the gap, we used cells proficient in excision repair (normal human fibroblasts) and cells deficient in excision repair (Chinese hamster V-79 and xeroderma pigmentosum group C) and employed three techniques to investigate repair: unscheduled DNA synthesis measured autoradiographically, the photolysis of bromodeoxyuridine (BrdUrd) incorporated into parental DNA during repair, and loss of sites sensitive to an endonuclease specific for pyrimidine dimers, each at saturation doses of UV and AAAF.

MATERIALS AND METHODS

Cell Culture

The cell types used were: normal human fibroblasts Rid Mor (CRL 1220) and xeroderma pigmentosum group C—Ge Ar (XP C, CRL 1161)—from the American Type Culture Collection (Rockville, Md.); another cell line of normal human fibroblasts (GM 498) from the Institute for Medical Research (Camden, N.J.) obtained from R. R. Tice (Medical Department, Brookhaven National Laboratory); and Chinese hamster V-79 cells obtained from M. Bender (Medical Department, Brookhaven National Laboratory). Cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 400 μg/ml L-glutamine, 140 U/ml penicillin, and 140 μg/ml streptomycin (Grand Island Biological Co., Grand Island, N.Y.) and kept in humidified 10% CO^2 atmosphere at 37°C. When cells reached confluency they were subcultured at a ratio of 1:3. Passages used were between 3–24 in human cells. Cells were regularly checked for the absence of mycoplasma contamination by the improved modification of fluorescent staining with bisbenzimidazol compound 33258 Hoechst (23; Hoechst Pharmaceutical Co., Kansas City, Mo.)

Unscheduled DNA Synthesis (UDS)

24 h before treatment, cells were seeded at a density of 10^3 cells/cm^2 onto four 11 × 22-mm cover slips. 1 h before treatment, hydroxyurea (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 4 mM to inhibit scheduled DNA synthesis (24). At zero time (time of treatment) cells were exposed to 20 Jm^{-2} of 254 nm UV at a dose rate of 0.72 Wm^{-2}, or for 20 min to 20 μM AAAF (a gift from J. A. Miller, McArdle Cancer Center, University of Wisconsin, Madison, Wis.) dissolved in fresh $(CH_3)_2SO$ (Fisher Scientific Co., Fair-

lawn, N.J.). In some cases cells were exposed to combined treatments with both agents as described previously (6, 17). To study the rate of unscheduled synthesis [^{3}H]deoxyribosylthymine (dThd), 2 or 5 μCi/ml (6.7 Ci/mmol, New England Nuclear, Boston, Mass.) was added to cultures of normal human and XP C cells, respectively, for 3 h; alternatively, 5 μCi/ml was added to V-79 for 1 h beginning at various times after treatment. Cells were coated with Kodak NTB photographic emulsion (Eastman Kodak Co., Rochester, N.Y.) for 4, 6, and 4 days for normal, XP C, and V-79, respectively, before being developed and analyzed as described before. If UDS was not to be measured immediately after treatment, cells were incubated in the absence of hydroxyurea until 1 h before the addition of [^{3}H]dThd, at which time hydroxyurea was added to give 4 mM.

BrdUrd Photolysis

Details of this technique and the concepts involved were previously described (8, 17). ^{3}H-labeled cells were treated and incubated in BrdUrd for several time intervals so as to mark the repaired regions in parental DNA. The repaired regions were selectively photolyzed by 313 nm radiation, and the resulting decrease in weight average molecular weight, M_w, of parental DNA measured by sedimentation in alkaline sucrose. To improve the precision, ^{14}C-labeled cells were treated in the same way, except they were permitted to repair in dThd, and coirradiated and cosedimented with ^{3}H-labeled cells. The difference $(1/M_w)_B - (1/M_w)_T = \Delta(1/M_w)$ as a function of 313 nm dose is a measure of the characteristics of total repair. 313 nm radiation emitting from a Johns monochromator (25) was used (courtesy of J. Sutherland, Biology Department, Brookhaven National Laboratory). In actual experiments, the dose rate was measured with a calibrated photocell whose output multipled by time could be expressed in breaks per BrdUrd residue. Typical dose rates were 3.3×10^2 Wm^{-2} corresponding to 7.5×10^{-3} breaks/BrdUrd per min.

Endonuclease-Sensitive Sites Assay

In this technique pyrimidine dimers are estimated from the numbers of single-strand breaks introduced into extracted DNA by a crude extract of *Micrococcus luteus*. The number of breaks was obtained from the number average molecular weight, M_n, calculated from $\frac{1}{2}$ (weight average molecular weight, M_w) measured by sedimentation in alkaline sucrose (18). To measure small changes in the numbers of endonuclease-sensitive sites per dalton, we usually mixed ^{3}H-labeled cells that were UV irradiated and incubated to permit excision to take place with ^{14}C-labeled cells that were irradiated with the same dose but not incubated. The difference $(1/M_n)_C - (1/M_n)_H$ represents the number of dimers removed per dalton. Details of the technique and the modifications required to achieve higher sensitivities using a *M. luteus* extract with low nonspecific endonucleolytic activity (<0.1 breaks/10^8 daltons) were described earlier (6, 17).

RESULTS

Unscheduled DNA Synthesis (UDS)

The results of measurements of UDS in cells treated with UV, AAAF, or a combination of the two are presented in Fig. 1. Fig. 1 A shows the amount of UDS per 3-h interval in normal human cells exposed to saturating doses of UV or AAAF. The rate of UDS is constant for the first 9 h; and from 12–24 h it is about three-fold lower for both UV and AAAF. Our measurements showed that the number of grains/nucleus were

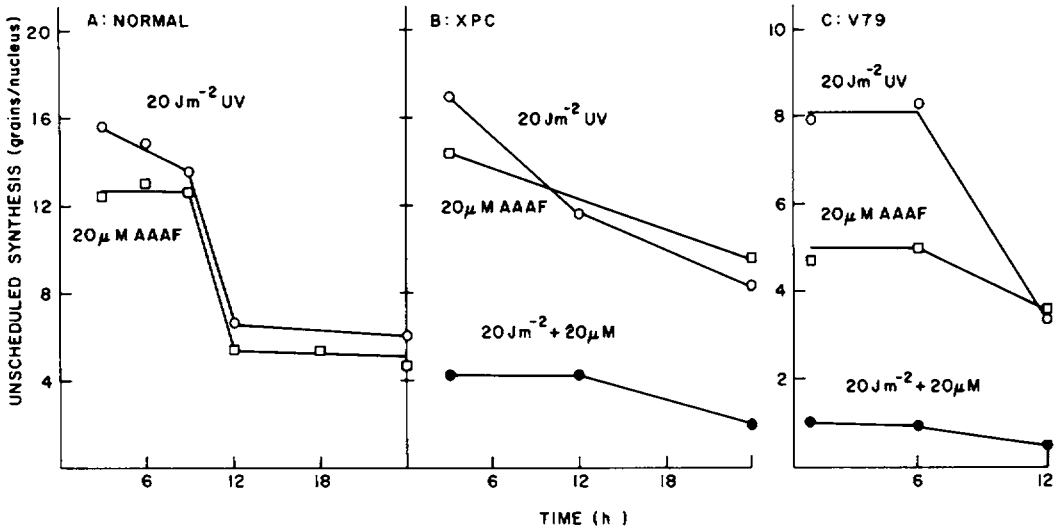

FIGURE 1 The amount of UDS, expressed as average number of grains per nucleus, as a function of time at which autoradiographs were made after cells were treated with UV, AAAF, or a combination of the two. (A) Normal human (CRL 1220). UDS measured for 3 h—2 μCi/ml [^{3}H]dThd added—and cells were exposed to photographic emulsion for 4 days. (B) XP C (CRL 1161). UDS measured for 3h—5 μCi/ml [^{3}H]dThd added—and cells were exposed to photographic emulsion for 6 days. (C) V-79 cells. UDS measured for 1 h—5 μCi/ml [^{3}H]dThd added—and cells exposed to photographic emulsion for 4 days.

13.6 and 12.6 for UV- and AAAF-treated cells at 9 h; whereas it dropped to 6.6 and 5.4 at 12 h for UV and AAAF, respectively. We believe that our results are accurate within 20%, which is the sensitivity of this technique. The initial rate of UDS for XP C cells (Fig. 1 B) is appreciably less than for normal human cells (the autoradiographic times and [^{3}H]dThd concentrations in Fig. 1 B were 1.5- and 2.5-fold greater than in Fig. 1 A), but the decrease in rate with time seems less than for normal cells. Moreover, at all times the rate of UDS from a combined treatment of UV plus AAAF is less than from either treatment alone. The latter finding also holds for V-79 cells (Fig. 1 C). In these cells the rate of repair of UV damage seems constant for the first 6 h, followed by a decline during the last 6 h, but AAAF repair does not seem to shut off as rapidly.

BrdUrd Photolysis

Data on the photolysis of BrdUrd incorporated into DNA during early and late repair intervals in normal human and V-79 cells are presented in Figs. 2 and 3. In normal human cells there is less repair of both UV and AAAF damage during the 15–27 h repair interval compared with the 0–12-h interval (Fig. 2). Note that the amount of repair after 20 μM AAAF appears to be appreciably less than for the 20 Jm^{-2} UV, though both doses seem to give approximately the same amount of UDS. We rule out the possibility that this variation may be due to a difference in patch size, because our

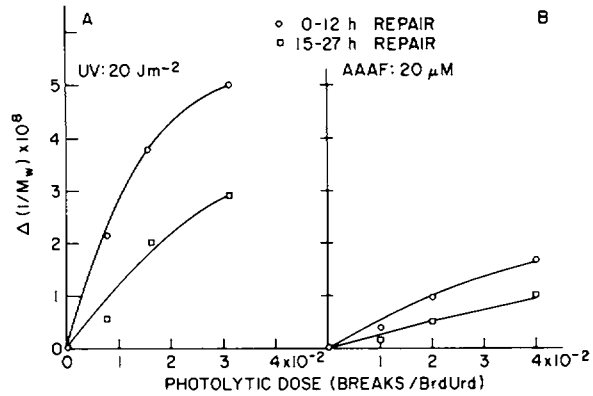

FIGURE 2 Results of the photolysis of BrdUrd by 313 nm radiation expressed as $\Delta(1/M_w)$ vs. photolytic dose for normal human cells (CRL 1220 and GM 498) exposed to 20 Jm^{-2} UV (A) or 20 μM AAAF (B). (o) Repair for 0–12 h; (□) 15–27 h.

experiments on patch sizes in normal human cells have shown an average UV patch size of 130 ± 60 and an AAAF patch size of 100 ± 70 (26). A possible explanation is that products of the interaction between AAAF and DNA remain bound to DNA for a long time and have appreciable absorbance at 313 nm, thereby sensitizing the DNA to radiation and to subsequent fragmentation in alkali (26, 28). Such fragmented DNA sediments near the top of the gradients and these radioactive profiles limit the resolution of the technique. Therefore, in subsequent experiments we used lower concentrations of AAAF (10 μM) and 10 μM AAAF in combination with 20 Jm^{-2} in the BrdUrd photolysis technique (16, 17). In V-79 there is also a marked decrease in repair between 6–12 h compared with 0–6 h in cells treated with UV or AAAF, though the decrease is less in the latter than in the former (Fig. 3).

We did an experiment on normal human cells where we allowed for repair for various times after treating cells with 10 μM AAAF (Fig. 4 A). Repair, expressed as $\Delta(1/M_w)$ as a function of time is plotted (Fig. 4 B) for the highest photolytic dose used (3.72 × 10^{-2} breaks/BrdUrd). The BrdUrd photolysis results are consistent with

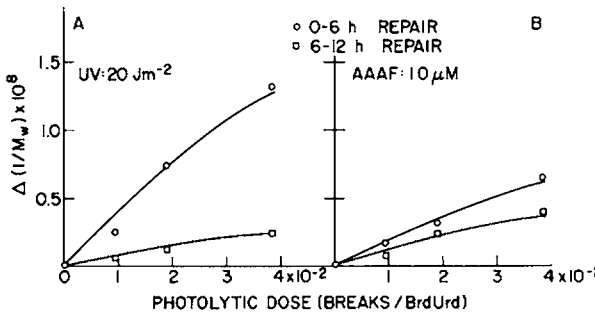

FIGURE 3 $\Delta(1/M_w)$ vs. photolytic dose for V-79 cells exposed to 20 Jm^{-2} UV (A) or 10 μM AAAF (B). (o) Repair for 0–6 h; (□) 6–12 h.

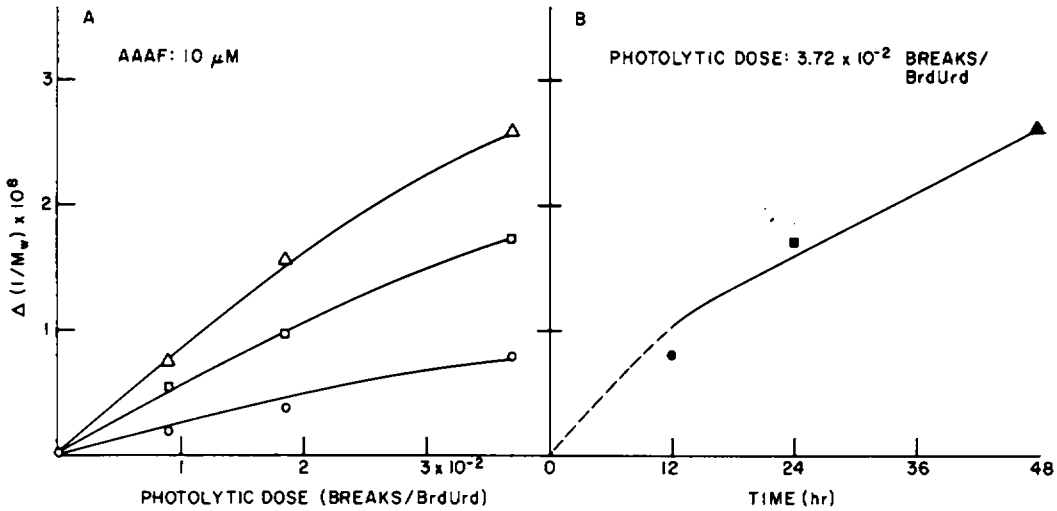

FIGURE 4 (A) $\Delta(1/M_w)$ vs. photolytic dose from normal human cells exposed to 10 μM AAAF. (○) Repair for 12 h; (□) 24 h; (△) 48 h. (B) The relationship between $\Delta(1/M_w)$ and time of repair for normal cells exposed to 10 μM AAAF and photolytic dose of 3.72×10^{-2} breaks/ BrdUrd.

the other repair measurements, but the data are not good enough to make a quantitative correlation.

Endonuclease-Sensitive Sites

We compared the number of endonuclease-sensitive sites removed in various time intervals after a UV dose of 20 Jm^{-2} to normal human cells and 5 Jm^{-2} to XP C cells. We showed earlier that in normal human cells a dose of 20 Jm^{-2} was saturating for the removal of endonuclease-sensitive sites in 24 h (16) and that in XP C cells the maximum number of endonuclease-sensitive sites removed in 24 h reached a plateau between 10 and 20 Jm^{-2} of UV (26). However, to get more accurate values of sites removed in XP C cells, we used a dose of 5 Jm^{-2}. The number of endonuclease-sensitive sites made by these doses, 52 and 13 sites/10^8 daltons, respectively, agree well with those obtained by Masker (29).

Fig. 5 shows typical alkaline sucrose gradients of DNA's, extracted from UV-irradiated cells, after treatment with UV-endonuclease. It is apparent that normal cells remove an appreciable number of endonuclease sites in 24 h whereas XP C cells do not. That is why we used a dose of 5 Jm^{-2} for XP C because higher doses would make it difficult to determine the small number of sites removed. The removal of sites as a function of time is shown in Fig. 6 A. In normal human fibroblasts there is a rapid removal during the first 6 h, followed by a steady rate of removal for the next 36 h. In XP C cells the rate of removal of sites was appreciably less than in normal human cells—in good agreement with autoradiographic data—and the rate seemed constant over the 48 h of the experiment. Fig. 6 B shows a summary of data similar to those in

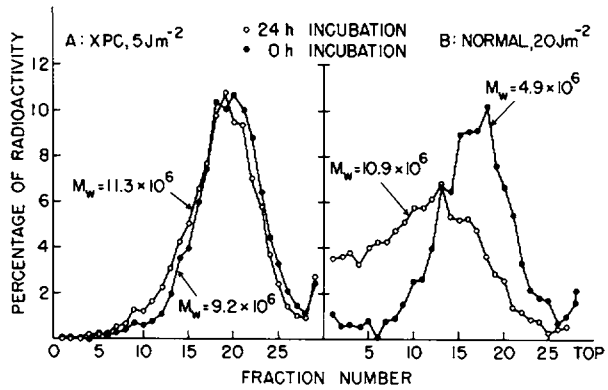

FIGURE 5 Sedimentation profiles of DNA after incubation with UV endonuclease. DNA labeled with [^{3}H]dThd was extracted 24 h after irradiation (○), or labeled with [^{14}C]dThd and extracted immediately after irradiation (●). Total cpm were 2,320 and 1,820 for cells labeled with ^{3}H or ^{14}C, respectively. (A) XP C cells treated with 5 Jm^{-2} of UV. (B) Normal human cells treated with 20 Jm^{-2} of UV. Total cpm were 1,610 and 710 for cells labeled with ^{3}H or ^{14}C, respectively. Sedimentation was done in an SW60 rotor at 50,000 rpm for 75 and 120 min, respectively.

Fig. 6 A for different UV doses. At any dose from 5 to 40 Jm^{-2}, XP C cells remove a smaller fraction of the sites in 24 h than do normal cells and, as earlier experiments showed (16), the percentage of sites removed in 24 h decreases with dose. The ratio of sites excised in XP C cells compared with normal human cells also decreases with dose. For example, at 5 Jm^{-2} it is 57%, whereas at 40 Jm^{-2} it drops to 25%.

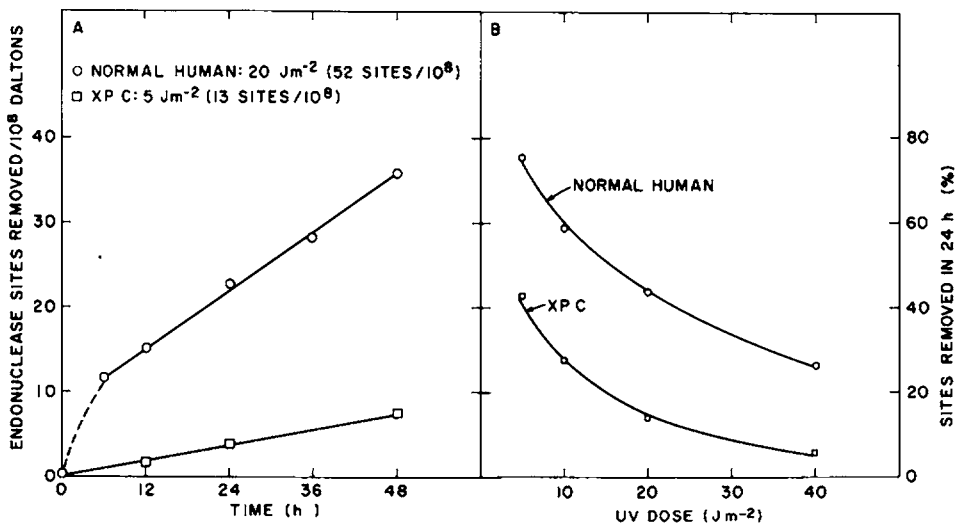

FIGURE 6 (A) The number of endonuclease-sensitive sites removed after 20 Jm^{-2} of UV radiation in normal human fibroblasts (○), and 5 Jm^{-2} of UV to XP C (□) as a function of time. (B) The percentage of endonuclease-sensitive sites removed in 24 h vs. UV dose in normal human (○) and XP C (□) cells.

DISCUSSION

Previous studies on repair kinetics of UV- and AAAF-treated mammalian cells were mostly concerned with excision repair-proficient cells. Doses employed were generally below saturation, and the kinetics resulting from combined treatments of both agents were not investigated. Hence, we used in this study three types of mammalian cells. Two of them were of human subjects—both proficient (normal) and deficient (XP C) in repair of both agents—and one was a Chinese hamster line (V-79) shown to be defective in repair of both agents (6, 16, 17). We investigated the kinetics of repair resulting from combinations of both agents and employed three techniques to measure repair: UDS, BrdUrd photolysis, and a UV-endonuclease assay. All techniques gave similar conclusions.

Several repair techniques have been used to show that in mammalian cells there is an approximate linear relationship between repair synthesis and time in the first few hours after irradiation (5, 18–22). The carcinogen AAAF has been shown to mimic UV in its repair characteristics (8, 13–15). Repair during the first 6 h was linear with time in human ammion AV_3 cells (5), whereas in WI-38 cells treated with AAAF there is a 30-min lag before the rate became linear (21). Our UDS data on cells proficient or deficient in excision repair, treated with saturation doses of UV and AAAF, show that in the first 6 h the repair rate is approximately constant. The kinetics of repair for the 24-h interval seem to exhibit slight variation from one cell line to the other. They all show a decrease with time, but the magnitude of the decrease differs. For example, in normal cells there is a threefold decrease in the extent of repair as measured by UDS during the 24-h interval (i.e., the average number of grains/nucleus dropped from 15.8 to 6 and from 12.3 to 4.7 in UV- and AAAF-treated cells, respectively), whereas it is only about twofold in XP C cells (average number of grains/nucleus dropped from 17 to 8.2 and from 14.4 to 10.6 in UV- and AAAF-treated cells, respectively). Trosko and Yager (5) found a 60–70% decrease in repair in AV_3 cells exposed to 5 JM^{-2} of UV and 7 μM AAAF 24 h after treatment. Poirier and DeCicco (21) reported that for WI-38 treated with 4.5 Jm^{-2} or 10 μM AAAF (removed 4 h later from culture medium) there was an 80 or 50% decrease in repair, respectively, 24–28 h after treatment. Amacher et al. (30, 31) have shown that about 50% of [^{14}C]AAAF remained bound to DNA 48 h after treatment, whereas removal of dimers was nearly complete at the same interval. BrdUrd photolysis data agree with the UDS data and show that in both normal human and V-79 cells there is less repair in later time intervals in both UV-irradiated and AAAF-treated cells. Our data agree qualitatively with those of Ehmann et al. (32), who measured UDS in human cells exposed to UV, and with those of Williams and Cleaver (33), who measured repair replication and loss of sites sensitive to T4 endonuclease V in UV-irradiated African green monkey kidney cells.

The decrease in repair cannot be attributed to the cytotoxic effects of high UV doses or AAAF concentrations on cells because Poirier and DeCicco (21) have shown similar repair kinetics between doses of 1 and 10 μM AAAF producing, respectively, 0.1 and 60% lethality (15). In our experiments involving treatment of radioactive cells with

saturation doses of UV, AAAF, or a combination of the two, we observed in 36 h no decrease in the amount of radioactivity compared to untreated cells. Nor did we observe any appreciable sloughing of cells from Petri dishes during the same interval. Further experiments have shown that the amount of repair in both normal and XP C cells does not increase between 20 and 80 Jm^{-2} or between 20 and 80 μM AAAF, indicating that higher doses do not affect repair (26). However, in XP C cells it is the combination of 20 Jm^{-2} and 20 μM AAAF that results in less repair than that observed for the agents separately. Cell viability studies employing trypan blue showed that at the highest doses used (20 Jm^{-2}, 20 μM AAAF, and 20 Jm^{-2} + 20 μM AAAF) both normal human and XPC cells excluded the dye to the same extent as the untreated controls 3 and 24 h after treatment (26). Our data show no shutoff of repair after exposure to such high concentrations of AAAF. Thus the inhibitory effects observed in repair-deficient cells when both agents were combined are not artifacts due to cellular lethality or a hypothetical shutoff of repair system by AAAF.

The data on endonuclease-sensitive sites in UV-irradiated normal human cells and BrdUrd photolysis data on AAAF-treated normal human cells indicate that the initial rate of repair during the first 6 h is rapid and is followed by a slower, steadier rate of repair for the next 36 h. Similar observation was reported by Paterson et al. (18), who showed a biphasic nature in the curve for site removal at certain doses— ≥ 12.5 Jm^{-2}— in normal AH cells; by Edenberg and Hanawalt (19) during the first 6 h in HeLa cells exposed to UV; by Porier and DeCicco in WI-38 cells exposed to either UV or AAAF (21); and by Cleaver et al. (22) in bovine cells.

The present data are not good enough to imply a difference in UV repair kinetics in normal and XP C cells during the first 6 h. The rapid repair at early times seems to be related to the fact that some parts of DNA are more accessible to repair enzymes than others (34).

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