13th Jerusalem Symposium on Carcinogenesis:
Fundamental Mechanisms & Environmental Effects
April 28-May 2, 1980

BNL 27643
BIO 3581

CONF-800476--1

DNA REPAIR IN HUMAN CELLS EXPOSED TO COMBINATIONS OF CARCINOGENIC AGENTS

R. B. Setlow and Farid E. Ahmed[1]
Biology Department, Brookhaven National Laboratory, Upton, New York 11973 USA

Normal human and XP[2] fibroblasts were treated with UV plus UV-mimetic chemicals. The UV dose used was sufficient to saturate the UV excision repair system. Excision repair after combined treatments was estimated by unscheduled DNA synthesis, BrdUrd photolysis, and the loss of sites sensitive to a UV specific endonuclease. Since the repair of damage from UV and its mimetics is coordinately controlled we expected that there would be similar rate-limiting steps in the repair of UV and chemical damage and that after a combined treatment the total amount of repair would be the same as from UV or the chemicals separately. The expectation was not fulfilled. In normal cells repair after a combined treatment was additive whereas in XP cells repair after a combined treatment was usually less than after either agent separately. The chemicals tested were AAAF, DMBA-epoxide, 4NQO, and ICR-170.

INTRODUCTION

Skin cancer is the most common cancer in the United States and experimental and epidemiological data indicate that it arises from the shorter UV (290-320 nm) that penetrates the stratospheric ozone layer and reaches the surface of the earth (Scott and Straf). The knowledge that cells from individuals with the disease XP are defective in one or more pathways for repairing UV damage to DNA (Cleaver and Bootsma; Setlow, 1978; Arlett and Lehmann; Friedberg et al.) reinforces the idea that damages to DNA are initiating events in skin carcinogenesis. A comparison of the dose-response curve for skin cancer incidence in the white population of the United States and in XPs indicates that DNA repair is effective in removing > 85% of UV damage from the average person and that as a result of this removal the skin cancer incidence is 10^2-10^4-fold less than in XPs (Setlow, 1980). Such estimates are very crude because of heterogeneity in both the average and in the XP populations, not only in their life-style and in the UV transmission of their skin, but in their repair capabilities. For example, there are seven different

complementation groups of XP and they vary widely in their abilities to repair UV damage (Friedberg et al.). Nevertheless, the calculation indicates that relatively small changes in repair might have large effects on cancer initiation.

It is important to know the times of repair compared to replication and transcription since damage to DNA will only have biological consequences if the altered template is enzymatically read. The fixation of mutations will depend on the relative time of replication compared to DNA repair (Maher, et al.) and the cytotoxic action of UV on fibroblasts could well involve transcriptional events that result in the synthesis of aberrant proteins which would not permit cells to remain attached to their substrate (Kantor and Hull). Hence, much experimental effort has gone into the measuring the kinetics of DNA repair. To do so properly it is necessary to know what alterations are being repaired and whether the alterations would have severe biological consequences if not repaired. For example, N-7 alklyguanine seems to be an innocuous damage whereas O^6 methylguanine is much more important biologically although it is numerically inferior to the N-7 product. UV irradiation of DNA results in the formation of many products (Setlow and Setlow) but one of these--pyrimidine dimers--can be shown to be a major lethal and mutagenic lesion in prokaryotic and eukaryotic systems by virtue of the fact that many of them contain photoreactivating enzyme. The enzyme binds to DNA and in the presence of light monomerizes the dimers and reverses the biological effect of UV. There is no other known substrate for the enzyme and hence it may be used as a diagnostic reagent for the role of pyrimidine dimers in biological effects. By this test dimers account for $\sim 80\%$ of the UV killing of frog cells in culture (Rosenstein and Setlow). Such data do not indicate the molecular mechanisms by which dimers affect cells.

Most laboratory experiments are carried out with UV of wavelength 254 nm but the effective wavelengths in sunlight seem to be closer to 305 nm (Setlow, 1974). The inference that dimers are as important at the longer wavelengths as at the shorter comes not only from the constancy of the photoreactivable sector as a function of wavelength (Rosenstein and Setlow) but from the shape of the action spectrum for killing mammalian cells which parallels closely that for the production dimers in both Chinese hamster cells (Rothman and Setlow) and in normal human and XP fibroblasts (Kantor, et al.). The latter argument is strengthened by the observation that the yield of other UV photoproducts relative to dimers increases dramatically at longer wavelengths (Cerutti and Netrawali). A further indication that 254 nm is a good model for the effects of many sunlight irradiations is the equality of the ratio of sister chromatid exchanges to endonuclease-sensitive sites in Chinese hamster cells irradiated by 254 nm and by simulated sunlight (Reynolds, et al.).

EXCISION REPAIR

Despite the fact that UV-induced pyrimidine dimers are well identified, major lesions, it is important to remember that both physical and chemical carcinogens make a multitude of products in DNA, for example, DNA protein crosslinks (Fornace and Little). The damage resulting from the physical agents is more or less randomly distributed along DNA, but chemical changes seem concentrated in the spacer regions between nucleosomes (Jahn and Litman). The removal of damages by excision repair is not completely random. It seems preferentially to begin in spacer regions but the data indicate that such regions are not static ones but seem to move along the DNA duplex (Smerdon et al.).The latter observations probably account for the changing kinetics of excision repair with time (see below).

The presumed sequence of steps in excision repair has been described many times (Hanawalt, et al.). Such descriptions show that an early step in excision repair involves attack on the damaged polynucleotide by an endonuclease such as a UV-specific endonuclease. Because few single strand breaks accumulate during repair and because fewer breaks are observed in excision-defective cells than in normally repairing ones, it seems as if the endonucleolytic step is the rate-limiting one. This point of view is reasonable but it should be remembered that the only UV-specific endonucleases that have been well characterized are those from T4 phage infected *E. coli* and from *M. luteus* (Paterson). These endonuclease preparations are specific for pyrimidine dimers and make one single strand nick per dimer. However, the *M. luteus* activity seems to be a combination of two separate ones--a glycosylase that splits half of the dimer from the polynucleotide (Grossman et al.) and an endonuclease acting at a later time (Setlow and Grist). The UV-endonuclease activity in *E. coli* seems to be a complex of proteins (Seeberg) and that from calf thymus is a large unstable protein (Waldstein, et al.). The fact that there are seven complementation groups of XP implies that any one of seven mutations results in a decrease in the ability to excise dimers and that many proteins or cofactors are associated with the endonuclease step in human cells.

The observations that many chemicals mimic UV (Regan and Setlow; Setlow, 1978; Friedberg, et al.) (see Table 1) suggests that there are similar rate-limiting steps for the repair of UV damage and damage from mimetics and that the chemical damages should compete for the repair system working on dimers in vivo. We have invest gated this competition.

In the work to be described three techniques were used to measure excision. They are: 1) UDS measured radioautographically by the incorporation of ^{3}HdThd for 3 hr after treatment; 2) the photolysis of BrdUrd incorporated into parental DNA for 12 hrs after treatment (Regan and Setlow); and 3) the loss during 24 hrs after treatment of sites in DNA sensitive to a UV endonuclease preparation

Table 1. Ways in which some chemical damages mimic UV damage in human cells.

1. UV- sensitive cells (XP) are more sensitive to the chemical than normal cells.
2. Chemically treated viruses show a higher survival on normal cells than on XP cells.
3. XP cells deficient in repair of UV damage are also deficient in excision of chemical damage.
4. Excision repair of UV and of chemical damage involves long patches (approx. 100 nucleotides).
5. XP complementation groups observed for repair of chemical damage are the same as those for UV damage (Zelle and Bootsma).

from M. luteus (Paterson, 1978). The first two techniques detect most types of excision repair and their quantitative values depend on the number of sites repaired, the patch size, and the concentration of thymine in the patches. The third technique measures only the loss of pyrimidine dimers and so is well suited to measure dimer repair in cells treated with combinations of UV and chemicals that mimic UV.

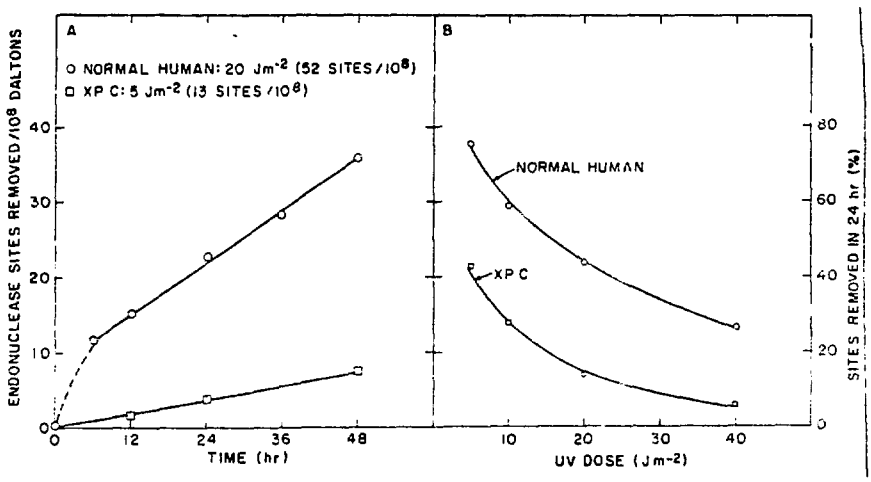

Figure 1. Excision repair as a function of time and dose for normal and XP fibroblasts (Ahmed and Setlow, 1978a).

The use of the endonuclease assay in measuring the excision of dimers is indicated in Fig. 1 which shows (A) excision versus time at a fixed dose for normal and XP cells and (B) the percentage excised at a fixed time for different initial doses. The latter figure shows not only the defect in excision in complementation group C of XP cells but indicates that the defect is not an absolute one and that because the repair system becomes saturated (see below) the fraction

of dimers excised decreases as the dose increases. Hence, at high doses it is difficult to measure repair by the loss of specific products. At such doses however, it is possible to measure repair easily by the other techniques.

If such experiments are to be done properly one must work at dose levels that saturate the repair systems. Figure 2 shows data for dimer removal versus dose for a number of cell strains. Not shown is the fact that the initial number of dimers increases

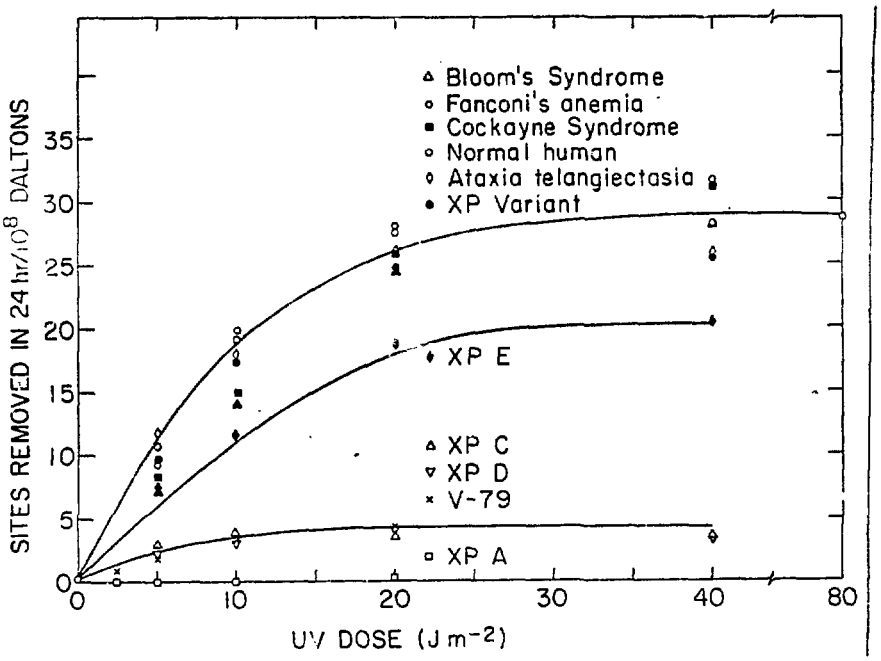

Figure 2. Endonuclease sensitive sites removed in 24 hrs as a function of initial UV dose for a number different human cell strains and Chinese hamster V79 cells (Ahmed and Setlow, 1979a).

proportionately with dose (Ahmed and Setlow, 1979b). Three conclusions are apparent from the data. 1) The repair rate in normal cells is saturated at doses of approximately 20 Jm^{-2} (approximately 50 sites per 10^8d) and at somewhat lower doses in XP and Chinese hamster cells. 2) XP cells are deficient compared to normal excising strains. 3) V79 cells are similar in their excision properties to XP C cells. Similar saturation data are observed, using the UDS and BrdUrd photolysis techniques for cells treated with UV or AAAF. The amount of UDS is consistent with the observed dimer removal and patch size (Ahmed and Setlow, 1979b). AAAF acted like UV if its concentration in μM equalled the UV dose in Jm^{-2}. We also obtained saturation data for human cells treated with an epoxide

of DMBA but for both normal and XP cells the saturation level was only 10-20% of that observed after UV (Ahmed, et al.). This observation in itself indicates that although the repair of UV and DMBA damage may be coordinately controlled, the repair pathways must be different.

COMPETITION BETWEEN UV AND ITS MIMETICS

Expectation.

Most of our work has been done at doses (20 Jm^{-2}) close to the

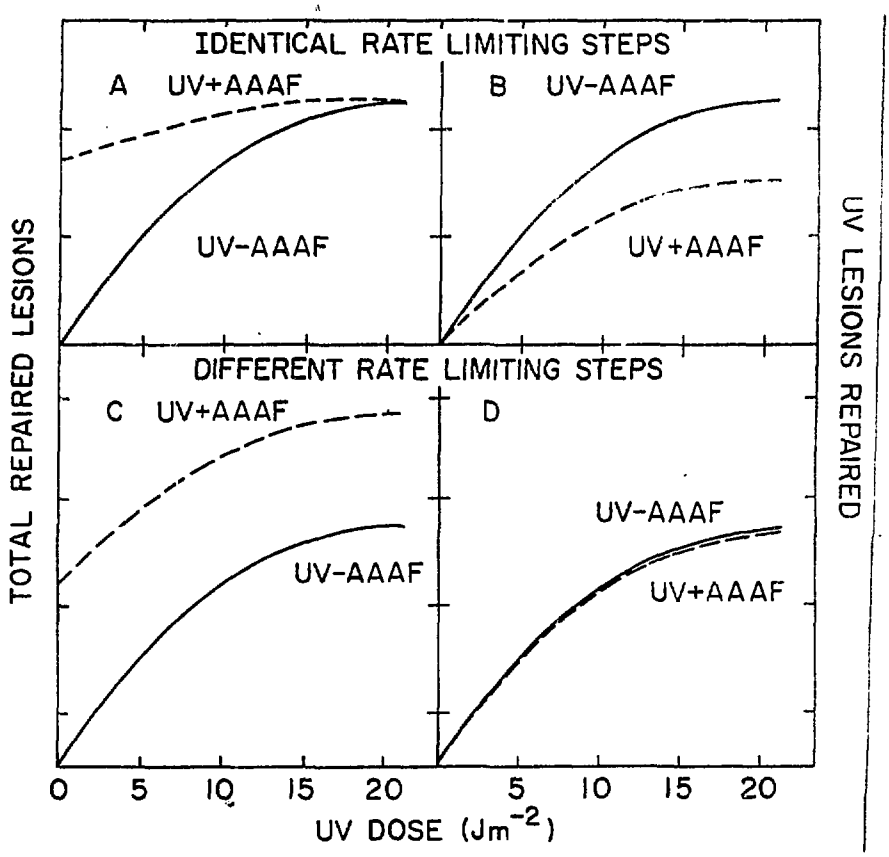

Figure 3. Possibilities for repair as a function of UV dose for cells treated ± 20 μM AAAF. (A and C): total repair; (B and D): dimers removed in 24 hr.

saturation level. Such doses are much greater than those received in sunlight. Noon sunlight in Texas would affect DNA in skin at the equivalent of a 254 nm dose rate of $\sim 0.01 Jm^{-2}$/min. The possible

results from a combined treatment with UV and one of its mimetics, AAAF, are shown in Fig. 3. Panels A and C give the possibilities for UDS or BrdUrd photolysis and panels B and D give the possibilities for the loss of endonuclease-sensitive sites. If the repair of AAAF damage had identical rate-limiting steps as UV repair, we would expect no increase in UDS and a decrease in dimer excision, since the repair would be shared between UV and AAAF lesions. If there were different rate-limiting steps, UDS would be additive and AAAF would not inhibit dimer excision.

UV PLUS AAAF

Typical UDS data are shown in Fig. 4. The results

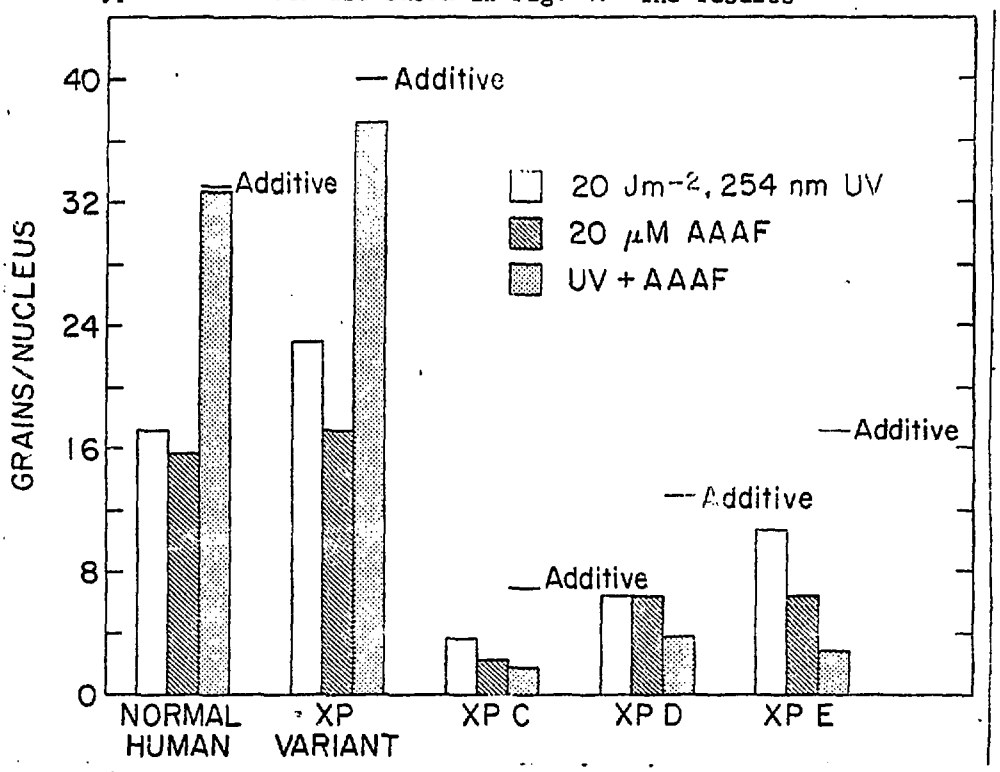

Figure 4. UDS in normal or XP cells exposed to UV or AAAF or the combination of the two (Ahmed and Setlow, 1979b).

indicate additivity of repair for normal excising strains and, not predicted in Fig. 3, an inhibitory effect for XP cells. The latter data cannot be explained by cell toxicity since the measurements were made on the plateau of the repair versus dose curve (see Fig. 2). Similar results using the other two assays and a number of other cell strains are shown in Table 2 (Ahmed and Setlow, 1978b, 1979b). The generalization is clear. AAAF damage does not compete with the

repair of UV damage in normal excising strains, but in XP cells each agent inhibits the repair of the other.

TABLE 2.

Measures of excision repair in human cells treated with UV, AAAF and combinations

Cell, line	Unscheduled synthesis(1)			Endonuclease assay(2)		BrdUrd photolysis(3)		
	$20Jm^{-2}$	20μM	$20Jm^{-2}$+20μM	$20Jm^{-2}$	$20Jm^{-2}$+20μM	$20Jm^{-2}$	10μM	$20Jm^{-2}$+10μM
Normal human								
Par Bel (CRL 1191)	18.6	16.1	33	27.5	27.4	3.5	1.4	4.9
Rid Mor (CRL 1220)	17.4	16.4	32.9	23.1	23.3	3.3	1.4	4.4
Ataxia telangiectasia								
NeNo (CRL 1347)	19.7	16.6	35	24.6	24.2	3.2	2.5	5.4
Se Pan (CRL 1343)	22.3	14	33.2	24.5	24.9	3.0	1.4	3.9
AT 4BI	19.6	14.4	35	26.3	26.4	2.2	1.4	3.4
Fanconi's anemia								
Ce Rel (CRL 1196)	15.4	13.9	28.2	27.5	27.4	3.4	0.9	4.4
Cockayne syndrome								
GM 1698	14.3	19.8	33.3	26.1	26.1	3.2	2.6	6.2
GM 1629				26.9	27.0	4.1	2.1	6.6
Xeroderma pigmentosum								
Variant: Wo Mee (CRL 1162)	23.0	17.1	37.2	24.7	24.0	2.8	1.1	4.3
C, Ge Ar (CRL 1161)	3.7	2.2	1.8	3.7	1.4	1.0	0.1	0.1
D, Be Wen (CRL 1169)	6.4	6.6	3.8	5.9	0.0	1.4	0.2	0.1
E, XP2RO (CRL 1259)	10.8	6.4	2.8	19.3	9.6	4.8	1.0	3.3

(1) Grains/Nucleus incorporated in 3 hr (8 days exposure).
(2) Sites removed in 24 hr/10^8 Daltons.
(3) $\Delta(1/M_n)\times10^4$ at highest 313 nm dose (12 hr repair).

UV PLUS 4NQO OR ICR-170

Repair after treatment of cells with 4NQO or ICR-170 is more complicated than after UV (Ahmed and Setlow, 1980). There is no well-defined saturation dose for these chemicals (Fig. 5). Presumably at high doses the chemicals damage proteins or membranes and as a result the amount of DNA repair goes down. We used concentrations well below the peak responses in Fig. 5 so there should be no possibility of a combined treatment exceeding the equivalent of the peak concentrations shown in Fig. 5. Hence, the results of the combined treatment are not as dramatic as those shown in Fig. 4 but, nevertheless, it is clear(Table 3) that UDS after combined treatments indicate additivity for normal human fibroblasts

Table 3. UDS in human cells treated with UV, ICR-170, 4NQO and combinations (Ahmed and Setlow, 1980).

	UV	ICR-170	Combined	UV	4NQO	Combined
normal	37.3	6.4	44.6	37.3	9.3	44.8
XP C	6.1	5.1	4.1	5.9	1.9	8.8

UV: $20Jm^{-2}$; ICR-170: 5 μM; 4NQO:0.5 μM

and an inhibitory effect for XP C fibroblasts treated with UV and ICR-170 but additivity for UV plus 4NQO. Similar results were obtained by the BrdUrd photolysis technique. A summary of our results is shown in Table 4.

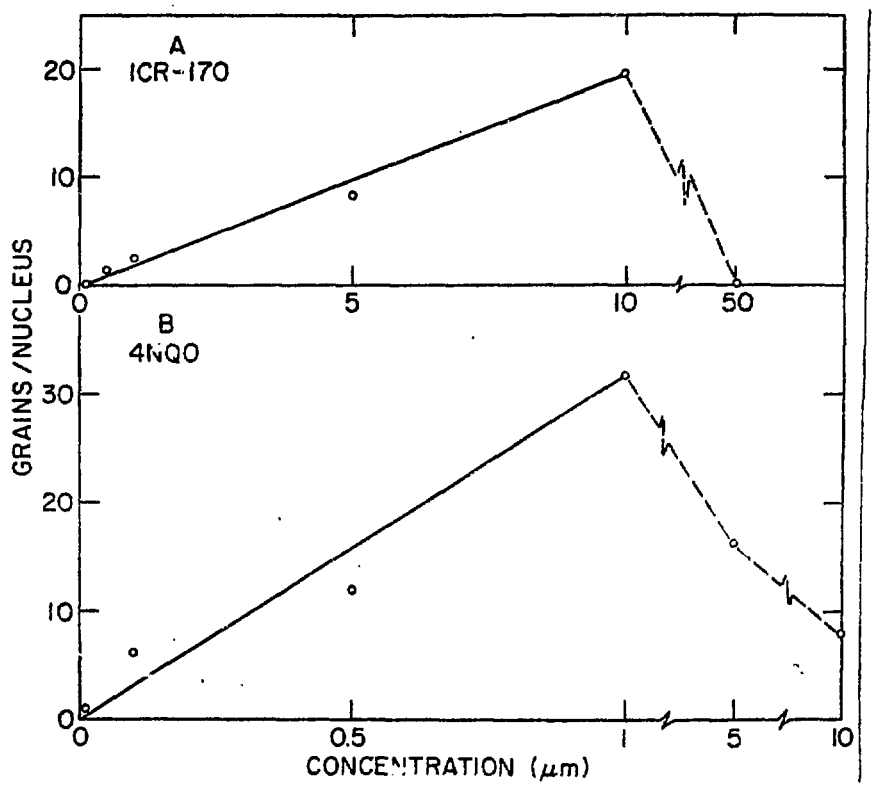

Figure 5. UDS in normal human fibroblasts versus concentration for ICR-170 and 4NQO (Ahmed and Setlow, 1980).

Table 4. DNA repair responses of human cells exposed to combinations of UV and its mimetics.

	Normal	XPC
UV + AAAF	additive	inhibitory
UV + DMBA-epoxide	additive	inhibitory
UV + ICR-170	additive	inhibitory
UV + 4NQO	additive	additive

Another group of investigators (Brown, et al.) has not observed additivity of repair in normal cells treated with UV plus AAAF or UV plus 4NQO. Since both their and our experiments seem definitive but contradictory, we suspect that they have not replicated our experiments in some as yet undefined way.

CONCLUSION

Obviously there is no simple explanation for all the observations we report. Nevertheless, we conclude that the repair pathways in XP cells are qualitatively different from those in normal human cells. If they were similar, but just had a lower level of the necessary enzymes or repair systems, we would expect that both groups of cells would either show additivity or an inhibitory effect. They do not. One could explain the results by hypothesizing that minor UV photoproducts might inhibit the repair enzymes in XP cells working on chemical damage but not inhibit those that work on UV damage and vice versa. Alternatively, one could construct rather elaborate models based on groups or complexes of proteins or cofactors to explain the observations that XP cells are defective in the repair of many damages to DNA but that normal cells do not have similar rate-limiting steps in the repair of these damages. For example Yarosh describes the possibility that there may be separate endonucleases for the different damages and that such endonucleases are normally present in relatively small numbers per cell. He further hypothesizes that the nucleases bind to DNA but do not nick it unless two or more cofactors common to all endonucleases associate with the nuclease bound to DNA. In normal cells there might be an excess of cofactors and the nucleases would be saturated with them and the repair would be additive. If the number of cofactors in XP cells was small, say 2- or 3-fold greater than the number of UV endonucleases, then after a combined treatment with UV and AAAF both UV and AAAF endonucleases would bind to DNA and the cofactors would be distributed equally between the two types of nucleases. As a result, only a small fraction of the nucleases would have two associated cofactors and the observed UV endonuclease activity would be depressed extensively as would be that of the putative AAAF endonuclease activity. These involved speculations are only presented to indicate the complexity of the problem and as a guide to further research.

ACKNOWLEDGEMENT

The research reported here was carried out at Brookhaven National Laboratory under the auspices of the U. S. Department of Energy.

REFERENCES

Ahmed, F. E., Gentil, A., Rosenstein, B. S., and Setlow, R. B.: 1980, Biochim, Biophys. Acta., in press.

Ahmed, F. E. and Setlow, R. B.: 1978a, Biophys. J. 24, pp. 665-675.

Ahmed, F. E. and Setlow, R. B.: 1978b, Biochim. Biophys. Acta 521, pp. 805-817.

Ahmed, F. E. and Setlow, R. B.: 1979a, Photochem. Photobiol. 29, pp. 983-989.

Ahmed, F. E. and Setlow, R. B.: 1979b, Cancer Res. 39, pp. 471-479.

Ahmed, F. E. and Setlow, R. B.: 1980, Chem.-Biol. Interactions, in press.

Arlett, C. F. and Lehmann, A. R.: 1978, Ann. Rev. Genet. 12, pp. 95-111.

Brown, A. J., Fickel, T. H., Cleaver, J. E., Lohman, P. H. M., Wade, M. H., and Waters, R.: 1979, Cancer Res. 39, pp. 2522-2527.

Cerutti, P. A. and Netrawali, M.: 1979, Radiation Research (Proc. 6th Int. Cong. Radiat. Res., Tokyo), pp. 423-432.

Cleaver, J. E. and Bootsma, D.: 1975, Ann. Rev. Genet. 9, pp. 19-38.

Fornace, A. J. and Little, J. B.: 1979, Cancer Res. 39, pp. 704-710.

Friedberg, E. C., Ehmann, U. K., and Williams, J. I.: 1979, Adv. Radiat. Res. 8, pp. 85-174.

Grossman, L., Riazuddin, S., Haseltine, W. A., and Lindan, C.: 1978, Cold Spr. Harb. Symp. Quant. Biol. 43, pp. 947-955.

Hanawalt, P. C., Cooper, P. R., Ganesan, A. K., and Smith, C. A.,: 1979, Ann. Rev. Biochem. 48, pp. 783-836.

Jahn, C. L. and Litman, G. W.: 1979, Biochemistry 18, pp. 1442-1449.

Kantor, G. J. and Hull, D. R.: 1979, Biophys. J. 27, pp. 359-370.

Kantor, G. J., Sutherland, J. C., and Setlow, R. B: 1980, Photochem. Photobiol., in press.

Maher, V. M., Dorney, D. J., Mendrala, A. L., Konze-Thomas, B., and McCormick, J. J.: 1979, Mutat. Res. 62, pp. 311-323.

Paterson, M. C.: 1978, Adv. Radiat. Biol. 7, pp. 1-53.

Regan, J. D. and Setlow, R. B.: 1974, Cancer Res. 34, pp. 3318-3325.

Reynolds, R. J., Natarajan, A. T., and Lohman, P. H. M: 1979, Mutat. Res. 64, pp. 353-356.

Rosenstein, B. S. and Setlow, R. B.: 1980, Photochem. Photobiol., in press.

Rothman, R. H., and Setlow, R. B.: 1979, Photochem. Photobiol. 29, pp. 57-61.

Scott, E. L. and Straf, M. L.: 1977, Origins of Human Cancer, Cold Spring Harbor Lab., pp. 529-546.

Seeberg, E.: 1978, Proc. Nat. Acad. Sc. USA 75, pp. 2569-2573.

Setlow, R. B.: 1974, Proc. Nat. Acad. Sci. USA 71, pp. 3363-3366.

Setlow, R. B.: 1978, Nature 271, pp. 713-717.

Setlow, R. B.: 1980, Arch. Toxicol.: in press.

Setlow, R. B. and Grist, E.: 1980, 8th Int. Photobiol. Cong., in press.

Setlow, R. B. and Setlow, J. K.: 1972, Ann. Rev. Biophys. Bioengineer. 1, pp. 293-346.

Smerdon, M. J., Kastan, M. B., and Lieberman, M. W.: 1979, Biochemistry 18, pp. 3732-3739.
Waldstein, E. A., Peller, S., and Setlow, R. B.: 1979, Proc. Nat. Acad. Sci. USA 76, pp. 3746-3750.
Yarosh, D.: 1979, Amer. Soc. Photobiol. Abstracts 7, p. 157.
Zelle, B. and Bootsma, D.: 1980, Mutat. Res., in press.

Footnotes

1. Present address: Pharmacopathics Research Laboratories, Inc., 9705 North Washington Blvd., Laurel, MD 20810.

2. Abbreviations used:

AAAF	- N-acetoxy-acetylaminofluorene
DMBA-epoxide	- 7, 12 dimethylbenz[a]anthracene 5, 6-oxide
ICR-170	- Acridine mustard
4NQO	- 4 nitroquinoline oxide
XP	- Xeroderma pigmentosum
UDS	- Unscheduled DNA synthesis
UV	- Ultraviolet