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MASTER

"DNA Repair in Mammalian Cells Exposed to Combinations of Carcinogenic Agents"

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Abbreviations: AAAF, N-acetoxy-2-acetylaminofluorene; DMBA-epoxide, 7,12-dimethylbenza[a]anthracene 5,6-oxide; ICR-170, acridine mustard; 4-NQO, 4-nitroquinoline 1-oxide; UV, ultraviolet; XP, xeroderma pigmentosum.

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ABSTRACT

Cells defective in one or more aspects of repair are killed and often mutagenized more readily than normal cells by DNA damaging agents, and humans whose cells are deficient in repair are at an increased carcinogenic risk compared to normal individuals. The excision repair of UV induced pyrimidine dimers is a well studied system, but the details of the steps in this repair system are far from being understood in human cells. We know that there are a number of chemicals that mimic UV in that normal human cells repair DNA damage from both these agents and from UV by a long patch excision repair system, and that xeroderma pigmentosum cells defective in repair of UV are also defective in the repair of damage from these chemicals. The chemicals we have investigated are AAAF, 4-NQO, DMBA-epoxide and ICR-170. The repair of UV and these chemicals seems to be controlled coordinately. We describe experiments, using several techniques, in which DNA excision repair is measured after treatment of various human cell strains with combinations of UV and these agents. If two agents have a common rate limiting step then, at doses high enough to saturate the repair system, one would expect the observed repair after a treatment with a combination of agents to be equal that from one agent alone. Such is not the case for normal human or excision deficient XP cells. In the former repair is additive and in the latter repair is usually appreciably less than that observed with either agent alone. Models that attempt to explain these surprising results involve complexes of enzymes and cofactors.

INTRODUCTION

There are estimates that 80-90% of human cancers are the result of environmental carcinogens or our life style. There have been many experiments attempting to detect environmental carcinogens by assays such as mutagenic assays using bacteria, carcinogenic assays with laboratory animals, and epidemiological studies. Such assays can indicate which agents are potentially dangerous to humans but they are not satisfactory for assaying the long-term effects of low level exposures. The latter problem is one that has defied a unique solution even for the effects of ionizing radiation. On the other hand, the effects of ambient ultraviolet (UV) in producing skin cancer have been assessed by epidemiological surveys in the United States (1). Part of the confidence in such assessment lies in the fact that a great deal is known about the molecular and cellular changes resulting from UV (2,3).

In a number of inherited human disorders the affected individuals are cancer prone (4,5 and other papers in this symposium). The prevalence of cancer among such individuals may be orders of magnitude higher than in the general population. Several of these disorders are associated with defects in the ability of cells to repair certain kinds of physical or chemical damage to their DNA. The identification of such disorders is direct evidence that damage to DNA can be carcinogenic and is the best available evidence for a causal connection between mutagenic and carcinogenic agents. The support for the connection is further

strengthed by the observation that mutations occur in xeroderma pigmentosum cells (XP) cells at lower doses of carcinogens than those affecting normal cells (6).

An analysis of the molecular defects in repair deficient diseases should give strong clues as to the molecular nature of the changes responsible for killing mutagenesis and carcinogenesis and hence give estimates of the probability that such changes result in biological effects. Since human risks are difficult to assess directly, they must be assessed from data on molecules and animals extrapolated to humans. The extrapolations will involve scanty epidemiological data. Hence, it is important that there be a good theoretical base for the extrapolation and that means an understanding of the molecular mechanisms invovled.

Almost all our ideas about the molecular nature of repair come from studies on bacterial systems because of the large number of well defined repair deficient mutants and the relative ease analyzing photochemical and molecular changes in them (2,3). In mammalian cells we have, at the moment, only the naturally occurring human inherited diseases. Hence, mammalian repair studies have taken their clues from the bacterial ones. Even in the bacterial world the problems are complex, alhough the concepts may be simple, because of the difficulty in purifing the first repair enzyme in the UV excision repair sequence - the so-called UV endonuclease (7). The endonuclease in E. coli contains several different proteins and recent evidence indicates that the endonuclease may also have a glycosylase activity associated with

it (8). In mammalian systems a number of endonucleases that attack UV irradiated DNA have been described but most of them do not act on pyrimidine dimers but on some minor and as yet undefined photochemical products. An endonuclease activity from calf-thymus glands has been described that does work on pyrimidine dimers, but the enzyme is very unstable (9).

Bacteria that are defective in repairing UV damage are also defective in repairing some chemical damages to their DNA. Hence, it is not suprising that the same thing is true for human cells and that the repair of damaged DNA is more general than just the special case of repair of pyrimidine dimers (2-5). There are a number of chemical agents whose damage in human cells seems to mimic those of UV in the ways indicated in Table 1.

It is apparent that the chemicals that mimic UV radiation are repaired by mechanisms that are coordinately controlled; that is, if cells are defective in UV repair they are defective in repairing these chemical damages. Since there are many experimental lines of evidence suggesting that the rate limiting step in excision repair of UV damage is the initial endonucleolytic step, early proposals to explain the results illustrated in Table 1 hypothesized that the rate limiting step - an endonuclease - was common for all these damages. Since human enzymes involved in excision repair are poorly characterized, it is not possible directly to compare the ability of such enzymes to repair chemical or physically damaged DNA. Hence, we have measured the ability of chemicals to compete for the UV repair system in human cells. Such experiments involve large doses, doses that

saturate the individual repair systems. Although such experiments give information about the rate limiting steps and the similarity of rate limiting steps of chemicals in UV, it is important to recognize that they are not directly applicable to the real world where people are also exposed to combinations of carcinogens, since in the latter case the doses and dose rates are much lower than those used experimentally.

METHODS

We have used three general methods to measure repair from individual agents or combinations of them. The first applies to UV damage and measures the loss of thymine containing dimers chromatographically or by the loss of sites sensitive to an endonuclease isolated from M. luteus that is specific for pyrimidine dimers (10). Since the endonuclease does not work on other types of damage it is easy to use it as a specific probe for UV damage and its repair in the presence of other types of damage. The second method is unscheduled DNA synthesis - the incorporation of label in treated cells during non-S phases of the cell cycle. Such measurements made radioautographically determine the repair of damage from chemical or ultraviolet or from combinations of agents. The third method is the photolysis of BrdU incorporated during repair. The technique give an estimate of the amount of BrdU incorporated and in this sense it is similar to unscheduled DNA synthesis, but the method has the advantage of giving an estimate of the size of the repaired patch (11). The three methods give consistent results for UV irradiated human cells; that is, the value of unscheduled DNA synthesis calculated

from the patch size and the numbers of dimers removed is consistent with that observed (12).

SATURATION OF REPAIR

Although the amount of UV damage increases with dose, at high doses the number of dimers excised in a given time is a constant. For example, at 20 J/m^2 (a dose that makes approximately one pyrimdine dimer/4 x 10^6D) the excision of dimers (measured as the percentage of radioactivity in dimers excised compared to thymine) is 0.050 whereas at 80 J/m^2 - a dose that makes four times as many dimers - it is 0.056 (13). Similar data are obtained for the loss of endonuclease sensitive sites (13). Figure 1 is an example of such saturation data. At high doses the number of endonuclease sensitive sites removed becomes constant and independent of dose. The repair system has become saturated. The various repair deficient XP cells strains also show saturation, but the numbers of sites removed in these strains is appreciably less than in normally excising strains. A second example of the saturation of DNA repair is shown in Fig. 2 where the amount of unscheduled synthesis in normal human cells is shown for cells treated with different concentrations of DMBA-epoxide (14). Although repair saturates for both UV and epoxide treatment, the saturation level is quite different for the two agents (see below). XP cells are defective in repair of both types of damage (see below) indicating a coordinate control of the repair of two types of damage but obviously the difference in the magnitudes of repair implies some more complicated mechanism than identical pathways for the repair of both types of damage. The

smaller amount of unscheduled synthesis for DMBA epoxide is not the result of a smaller patch size (14).

EXPECTATION FOR A COMBINATION OF AGENTS

The two extreme possibilities for the results of treatment of human cells with a combination of agents, such as UV and one of its mimetics, might arise from identical rate limiting steps for the agents or completely different rate-limiting steps for the agents. A schematic diagram of the expectation for each of these possibilities is shown in Fig. 3. Illustrated in the upper portion is the finding that the amount of damage increases proportionately with dose. The lower portions outline the expectations for different rate limiting steps (one would expect to find twice the amount of repair from either agent alone), or for identical rate limiting steps (the maximum amount of repair after treatment with two agents should be no greater than treated with one agent alone). For different rate limiting steps a UV mimetic should not interfere with the excision of dimers. For identical rate limiting steps a UV mimetic should inhibit the excision of dimers.

REPAIR AFTER UV PLUS AAAF

Fig. 4 shows the results of unscheduled synthesis experiments on several human cell strains treated with UV, with AAAF or with a combination of the two (12). The concentrations chosen are at saturating levels and it is apparent that in normal human cells repair after the combination is additive; whereas, in the XP strains the total repair after the combination is appreciably less than additive. Normal cells act as if the rate limiting step for

the repair of the two agents is completely different. In XP cells, however, the data indicate that each agent inhibits strongly the repair of the other so that after a combined treatment the total repair is appreciably less than the repair of either agent separately. The data can not be explained by general toxicity because, for example, high doses of UV do not inhibit UV repair nor do high doses of AAAF inhibit AAAF repair. We have obtained data leading to similar conclusions using the endonuclease sensitive site assay and the photolysis of BrdU incorporated during repair. A summary of these data (15) for a number of human cell strains is shown in Table 2. The generalization indicated above holds for all. Repair is additive in normal cells and strongly inhibitory in cells defective in excision repair.

REPAIR AFTER UV AND OTHER UV MIMETICS

Results very similar to those obtained for UV and AAAF are obtained for UV and other UV mimetics. For example, Fig. 5 shows unscheduled DNA synthesis data for normal human and XP cells treated with UV or DMBA-epoxide or a combination of the two (14). The doses used were saturating ones (see Figs. 1 and 2). Nevertheless, the maximum repair after chemical treatment is appreciably less than after UV exposure in both normal and XP cells although repair in XP cells is appreciably less than normal for both agents. For normal cells repair after a combined treatment is approximately additive whereas in XP cells repair is appreciably less than that observed for UV alone. Thus, UV plus DMBA-epoxide falls into the same category as UV and AAAF. A similar conclusion is reached using the other measures of DNA repair.

Similar experiments have been carried out for combinations of UV and 4-NQO and UV plus ICR-170 (16). The results of these experiments are summarized briefly in Table 3. In all the cases we have investigated the so-called UV mimetics do not inhibit repair of UV damage in normal human cells. Except in the case of 4-NQO the chemical agents inhibit UV repair in XP cells. Thus we reach a general conclusion that the low level of repair in XP cells is not the result only of a smaller number of enzymes involved in the rate limiting step. The fact that combined agents usually act as if they inhibit one another in XP cells, whereas they give an additive result in normal cells implies that the character of the rate limiting steps are different for normal and XP cells.

SPECULATIONS AND CONCLUSIONS

In the absence of direct knowledge of the enzymes involved in excision repair and a direct knowledge of their biochemical properties, anything we say is really speculative. Nevertheless, we can summarize some of the crucial evidence that leads us to hypothesize the existence of repair complexes in Table 4.

In normal human cells we might think of the repair complex as made up of all the kinds of enzymes that could participate in excision repair (glycosylases, nucleases, polymerases, ligases, proteases, etc). Each chemical damage, within rather broad limits, might have its own endonuclease associated with the other repair enzymes since it seems as if it is the endonuclease that has the specificity for repair. A change in any one of the enzymes in the repair complex, not necessarily the endonuclease,

would change the entire activity of the complex and the endonucleolytic activity might fail because of distortion of this multi-enzyme complex. Thus, even a change in an exonuclease might result in an observation of inhibition of endonucleolytic function. Such an explanation easily can explain the observations for normal human cells. It cannot, however, explain the data for XP cells indicating that the various UV mimetic agents often inhibit strongly UV repair so that the resulting repair from combined treatments is much less than that observed from either agent alone. Thus, one must construct more elaborate models to explain the XP results. For example, suppose there were separate endonuclease complexes for the different damages and that in XP cells each complex was present in relatively small numbers. Suppose further that although an endonuclease might bind to damaged sites in DNA, it might not nick them unless two or more cofactors bind to the nuclease itself after it is bound to DNA (18). Hence, in UV irradiated XP cells the small number of endonucleases would bind to DNA and the cofactors would bind to the nuclease and there would be a slow level of excision repair. If the number of cofactors were limited, then after a combined treatment with UV and AAAF the cofactors would distribute themselves among the different endonuclease complexes binding to AAAF and to UV damage. As a result the probability of two cofactors being associated with any one endonuclease complex would be very small. Hence repair would be much lower than expected. These involved speculations are only presented to indicate the

complexity of the problem and to illustrate the fact that they will only be solved by understanding more about the basic nature of the repair enzymes involved.

ACKNOWLEDGMENT

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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).

TABLE 2

MEASURES OF EXCISION REPAIR IN HUMAN CELLS TREATED WITH UV, AAAF AND COMBINATIONS

Cell line	Unscheduled synthesis			Endonuclease assay		BrdU photolysis		
	$20Jm^{-2}$	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 1098	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 Mec (CRL 1162)	23.0	17.1	37.2	24.7	24.9	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 1160)	6.4	6.6	3.8	3.9	0.8	1.0	0.2	0.4
E; XP2RO (CRL 1259)	10.8	6.4	2.8	19.3	9.6	4.8	1.0	3.8

[a] Grains/Nucleus incorporated in 3 hr (8 days exposure).
[b] Sites removed in 24 hr/10^8 Daltons.
[c] $(1/M_w) \times 10^8$ at highest 3131 nm dose (12 hr repair).

TABLE 3

REPAIR RESPONSES OF HUMAN CELLS

TO COMBINATIONS OF DNA DAMAGING AGENTS

	Normal Human	XP-C
UV + AAAF	additive	inhibitory
UV + DMBA-epoxide	additive	inhibitory
UV + ICR-170	additive	inhibitory
UV + 4-NQO	additive	additive

TABLE 4

WHY HYPOTHESIZE ENZYME COMPLEXES FOR
EXCISION REPAIR IN HUMAN CELLS?

a) UV mimetic chemicals exist (11).

b) XP cells of seven complementation groups (17) are defective in excision of UV damage and, where investigated, of damage from these chemicals.

c) Few single stand breaks accumulate during repair (18) and there seem to be many fewer single strand breaks in XP cells than in normal ones. Moreover, the addition of exogenous UV endonuclease to XP cells restores, in part, their UV repair activity (19).

d) Hence a change in any one of seven associated molecules changes the endonuclease function in repair and affects repair of many damges coordinately.

e) A possible explanation is that there is a universal repair endonuclease that is composed of seven or more subunits.

f) If were true, the repair pathways for UV and chemical damages would be the same and chemical treatment would inhibit UV repair at saturating doses. This is not the case.

FIGURE LEGENDS

Fig. 1. DNA repair in UV irradiated cells as a function of initial dose. Repair was measured as the loss in sites sensitive to a UV-endonuclease from M. luteus during 24 hr after irradiation. Endonuclease sensitive sites are equivalent to pyrimidine dimers (From ref. 13).

Fig. 2. DNA repair, measured as unscheduled synthesis during 3 hr, in normal human fibroblasts exposed to several concentrations of DMBA-epoxide in serum free medium for 30 min (From ref. 14).

Fig. 3. A schematic diagram illustrating the repair of damage as a function of dose for a combined treatment of cells with UV and AAAF. The lower part shows the expected results for two hypotheses about the rate limiting steps. The repaired regions are represented by ~~~~.

Fig. 4. Unscheduled DNA synthesis (the distribution of grains among cells) in normal human and in XP cells after treatment with saturating doses of UV, AAAF, or a combination of the two (From ref. 12).

Fig. 5. Unscheduled DNA synthesis (the average number of grains per nucleus) for normal human and XP cells exposed to UV, DMBA-epoxide, or to a combination of the two (From ref. 14).

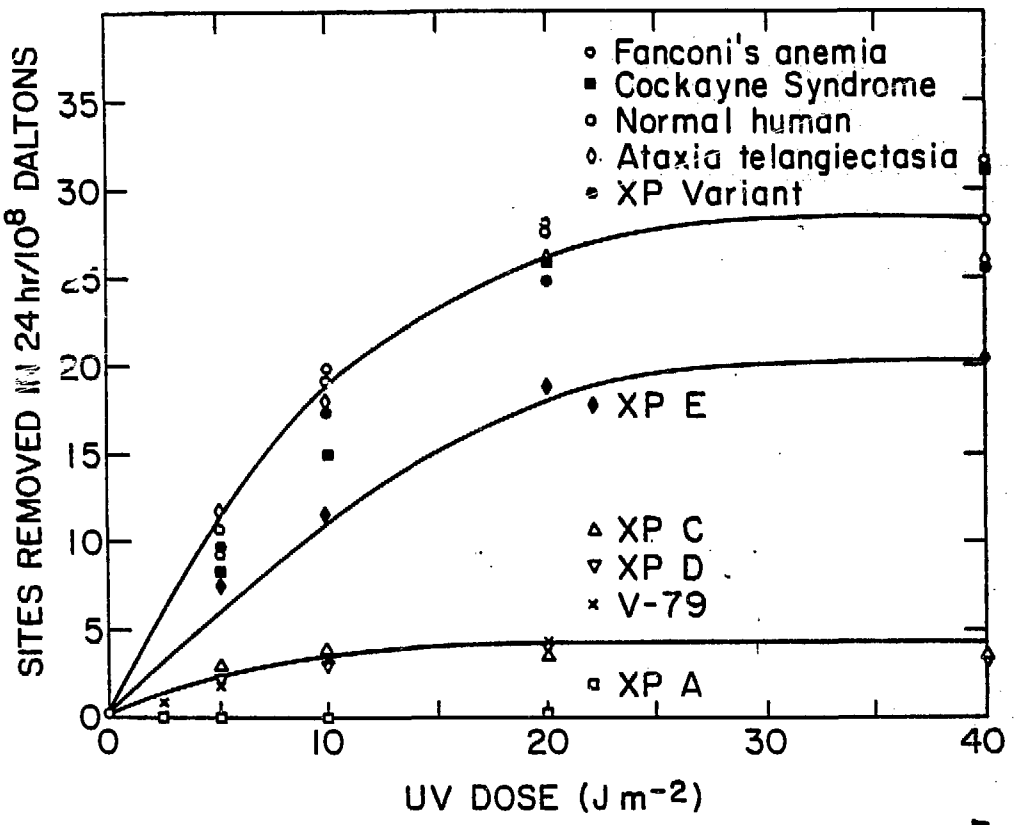
Fanconi's anemia
Cockayne Syndrome
Normal human
Ataxia telangiectasia
XP Variant
XP E
XP C
XP D
V-79
XP A
SITES REMOVED IN 24 hr/10^8 DALTONS
UV DOSE (J m^-2)
0
5
10
15
20
25
30
35
40

Fig. 1

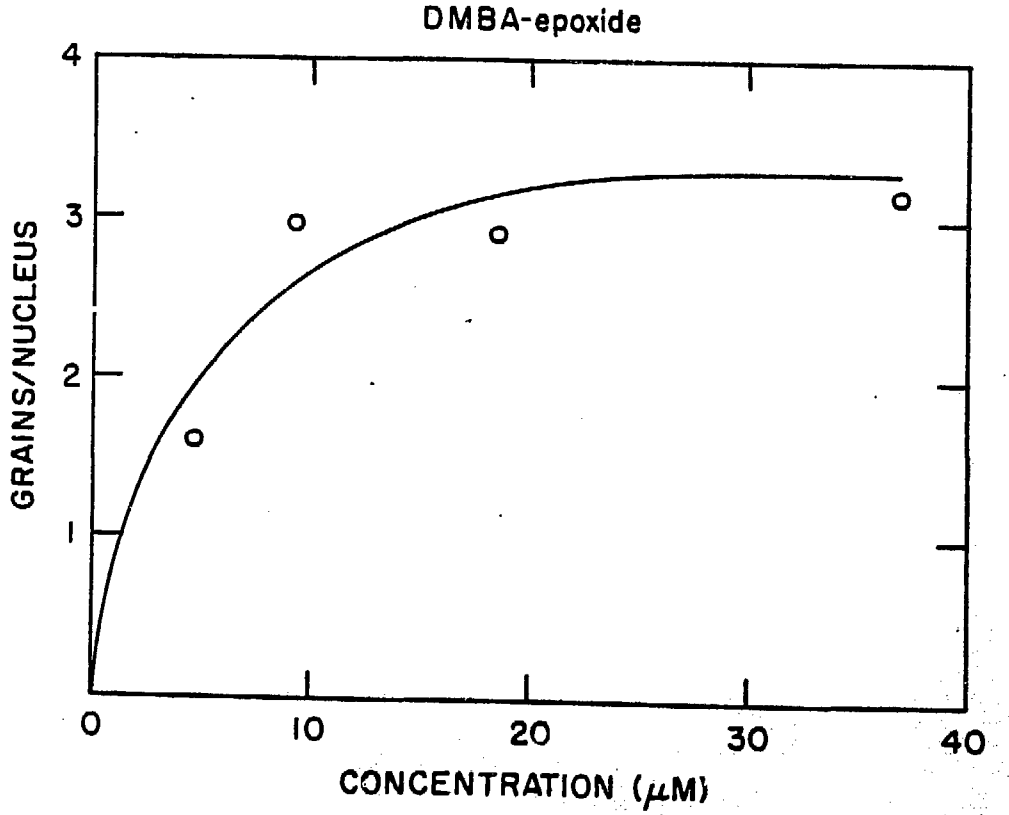
DMBA-epoxide
GRAINS/NUCLEUS
CONCENTRATION (μM)
0
1
2
3
4
10
20
30
40

Fig 2

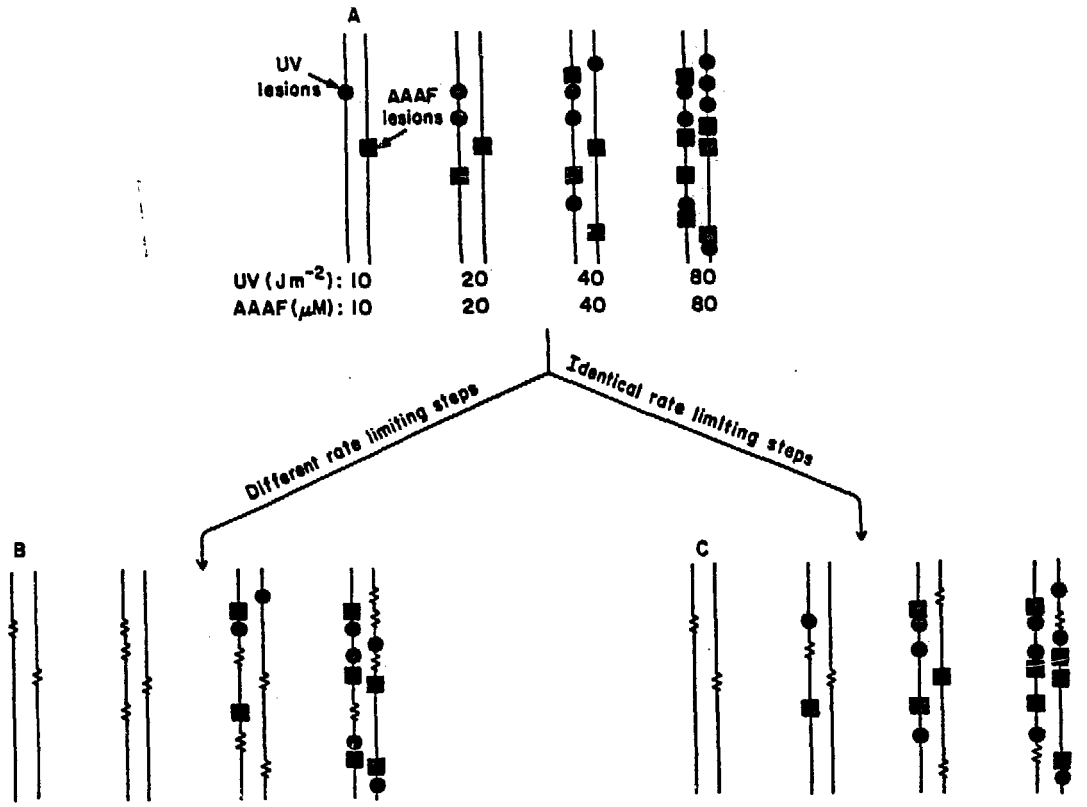
A
UV lesions
AAAF lesions
UV (J m⁻²): 10 20 40 80
AAAF (μM): 10 20 40 80
Different rate limiting steps
Identical rate limiting steps
B
C

Fig. 3

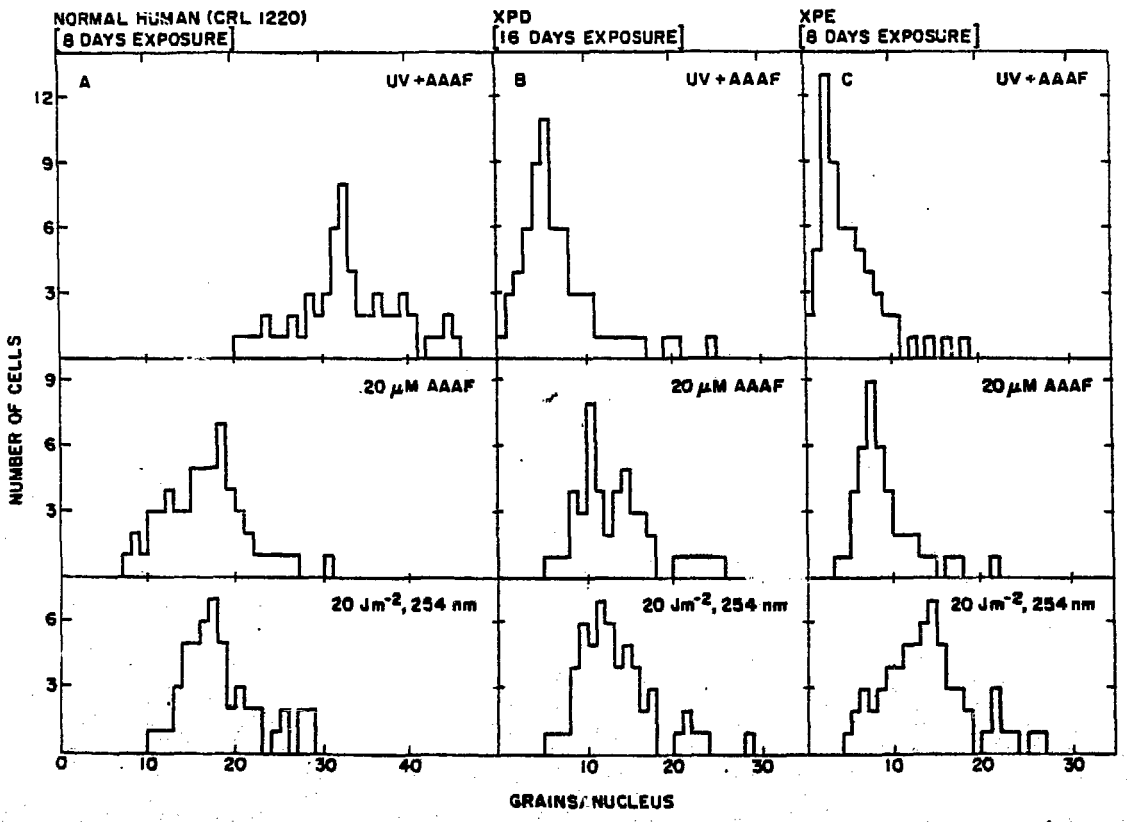
NORMAL HUMAN (CRL 1220)
[8 DAYS EXPOSURE]
XPD
[16 DAYS EXPOSURE]
XPE
[8 DAYS EXPOSURE]
A
B
C
UV+AAAF
20 μM AAAF
20 J m⁻², 254 nm
NUMBER OF CELLS
GRAINS/NUCLEUS

Fig. 4

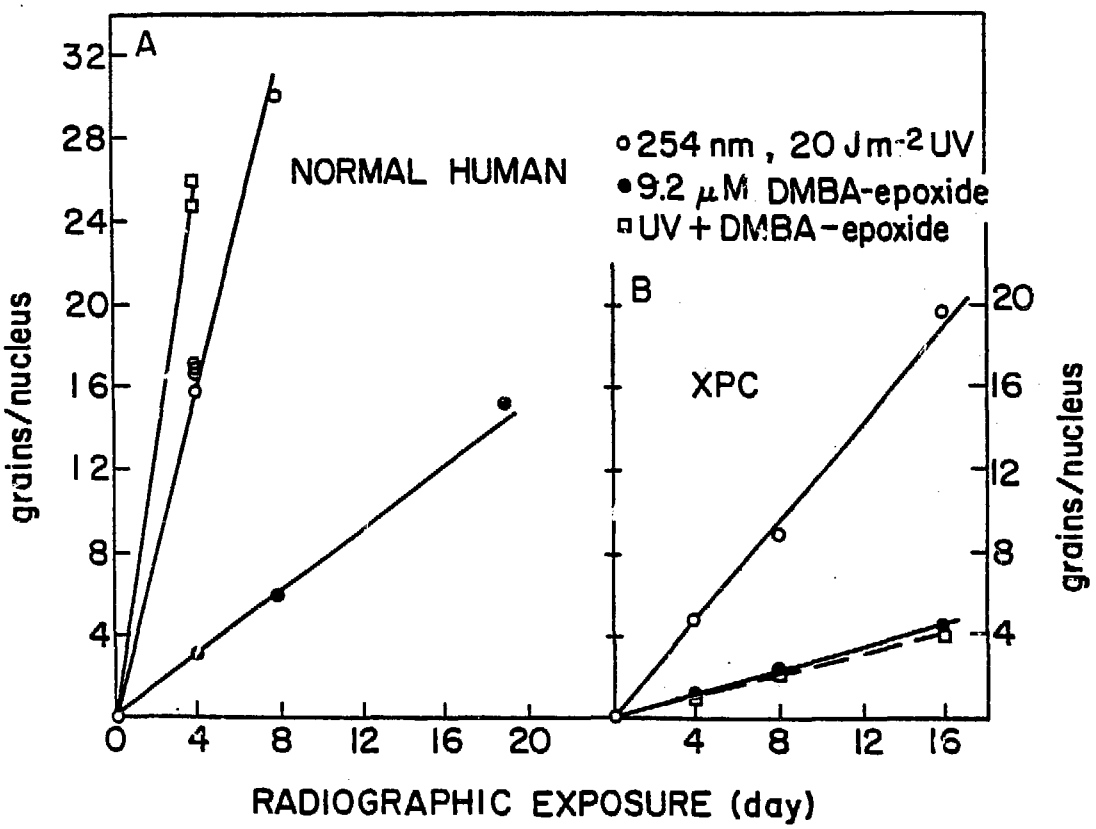
A
B
NORMAL HUMAN
XPC
○ 254 nm , 20 J m⁻² UV
● 9.2 μM DMBA-epoxide
□ UV + DMBA-epoxide
grains/nucleus
grains/nucleus
RADIOGRAPHIC EXPOSURE (day)
0
4
8
12
16
20
24
28
32

Fig. 5