

Evidence for Two Independent Pathways of Biologically Effective Excision Repair from Its Rate and Extent in Cells Cultured from Sun-sensitive Humans¹

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ABSTRACT

Repair-proficient human cells can be sensitized to exposure to UV radiation at 254 nm by postirradiation incubation in the presence of the eukaryotic α polymerase inhibitor, aphidicolin. The degree of sensitization has been examined in cells cultured from humans suffering from various types of sun-sensitive syndromes. Xeroderma pigmentosum (XP) variant and Bloom's cell lines (both excision proficient) were strongly sensitized by aphidicolin. An excision repair proficient Cockayne's cell line and a deficient XPD line were both sensitized to a level similar to the sensitivity of excision deficient XPA cells. In contrast, three XPC cell lines which show intermediate UV-induced repair replication and UV sensitivity were sensitized little (in one case) or not at all (in two cases) to UV by postirradiation inhibition of the α polymerase. These results lead us to conclude that there are two independent pathways of biologically effective excision repair, the major one of which involves the α polymerase and a second, less efficient and slower pathway which is independent of the α polymerase and which is the only pathway operating in two of the three XPC strains tested. The rates of biologically effective excision repair were similar in normal, XP variant, and Cockayne's cell lines, but these rates were considerably higher than published rates of dimer excision measured under similar conditions.

INTRODUCTION

Cells derived from sun-sensitive cancer-prone individuals with the disease xeroderma pigmentosum were shown several years ago to be defective in excision repair after exposure to radiation at 254 nm (1). The critical importance of excision repair for removal of lethal damage induced by far UV (254 nm) radiation in human fibroblasts has since been amply demonstrated by measurements which showed that the clone-forming efficiency of cells cultured from individuals with the classical forms of XP² (excision repair deficient) was much more UV-sensitive than that of cells derived from normal individuals (see, for example, Ref. 2). The several genetic complementation groups of the disease which have now been recognized are all believed to involve a defect at an early step of excision repair that involves damage recognition and/or incision (3). However, the different groups vary widely in their levels of UV-induced repair synthesis and UV sensitivity. Cells cultured from individuals with a variety of other sun-sensitive syndromes (XP variant, Bloom's, Cockayne's) are also UV sensitive to varying degrees, but they are not deficient in excision repair. The enzymology of the defect in all these syndromes remains unclear. All but Cockayne's are associated with enhanced susceptibility to cancer.

The discovery of aphidicolin, a drug which binds strongly but reversibly to the α polymerase (4), has provided a means to investigate the repair function of this critical enzyme in normal and UV-sensitive eukaryotic cells. Both biochemical (5-7) and biological (8, 9) studies using aphidicolin now indicate that the

α polymerase is the major polymerase involved in excision repair in both dividing and nondividing human fibroblasts so that aphidicolin is the most specific blocker of eukaryotic excision repair currently available. In the present study, we have exploited this fact in order to investigate the extent and rate of BEER that occurs in various UV-sensitive human cell lines.

MATERIALS AND METHODS

Cell Strains and Culture. All primary human skin fibroblast strains were originally obtained from the Medical Research Council Cell Mutation Unit (Sussex, England) except for XP12BE and GM 677 which were purchased from the Human Genetic Mutant Cell Repository (Camden, NJ). The strains and the designation of the syndromes suffered by their donors are listed in Table 1. Cell cultures were passaged at weekly intervals in Earle's modified minimal Eagle's medium (Gibco) supplemented with penicillin, streptomycin, sodium bicarbonate, glutamine, and 15% FCS.

UV Irradiation and Aphidicolin Treatment. To measure the extent of aphidicolin-inhibitable repair, cells were trypsinized, seeded into Petri dishes at the appropriate levels for the cloning assay, and incubated overnight (16-18 h) in 15% FCS. Dishes were washed with phosphate-buffered saline and irradiated with the appropriate fluence of UV (254 nm) from a germicidal lamp whose fluence rate was monitored by an International Light radiometer (Model IL770A) equipped with a calibrated SEE/400 probe and an interference filter (254 nm). Medium containing 0.5% FCS and either 0.25 or 1 μ g/ml of aphidicolin (ICI Pharmaceuticals) was then added to the plates which were incubated at 37°C for 48 h. These conditions have previously been shown to be optimal for measuring the inhibitable repair sector (8)³ and give maximum values (1 μ g/ml) at least in the low fluence region up to 6 Jm⁻². The cells are dividing at the time of the irradiation treatment. After the incubation period, plating efficiency was measured by adding 10 ml fresh medium containing 15% newborn calf serum and 6 \times 10⁴ lethally γ -irradiated (3.5 krad) fibroblasts to each plate and then incubating them for 17 days for clone formation.

The rate of repair was measured in arrested cells prepared, as described in detail previously (9), by seeding fibroblasts at subconfluent levels and incubating them for 10 days in medium containing 0.5% FCS. At the start of each experiment, dishes were UV or sham irradiated and then incubated for various periods in complete medium prior to a 48-h exposure to 1 μ g/ml of aphidicolin. For each experimental point, control or UV-irradiated cells were incubated for an equivalent period of time without aphidicolin in order to provide control values.

RESULTS

Extent of Aphidicolin-inhibitable Repair. A series of normal and of UV-sensitive primary fibroblast lines derived from patients with syndromes involving solar sensitivity have been exposed to graded fluences of UV and then incubated for a 2-day period in the absence or presence of aphidicolin (Figs. 1-4). As reported previously (8, 9), aphidicolin blocks BEER and markedly sensitizes repair proficient cells to UV treatment (Fig. 1, a and b). A UV-sensitive strain belonging to XP complementation group A is not sensitized by the drug treatment (Fig. 1c) consistent with a lack of measurable excision repair activity (see also Ref. 8). Cells derived from XP variant patients are reported

³ Unpublished results.

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² The abbreviations used are: XP, xeroderma pigmentosum; BEER, biologically effective excision repair; FCS, fetal calf serum; DHFR, dihydrofolate reductase.

TWO PATHWAYS OF EXCISION REPAIR IN HUMAN CELLS

Table 1 Sectors of aphidicolin-inhibitable repair in normal and mutant human fibroblast strains

Cell type	Designation	UV fluence to reduce survival to 10% (control) Jm^{-2} (C)	UV fluence to reduce survival to 10% (48 h incubation in 1 $\mu g/ml$ aphidicolin) Jm^{-2} (B)	Aphidicolin-inhibitable repair (fraction of total) ^a	Aphidicolin-inhibitable repair (fraction of excision repair) ^b
Normal	GM 730	8.8	2.9	0.67	0.71
	IBR/3	14.8	2.5	0.83	0.86
XPA	XP4LO	0.45 (A)	0.45	0	0
XP variant	XP30RO	7.1	1.3	0.82	0.87
	XP3DU	8.5	1.6	0.81	0.86
	XP6DU	9.1	1.7	0.82	0.86
Bloom's	GM1492	9.5	1.3	0.86	0.91
Cockayne's	CS698	2.4	0.65	0.73	0.90
XPC	XP4BR	1.7	1.7	0	0
	XP10LO	2.2	1.3	0.42	0.53
	GM677	3.2	3.2	0	0
XPD	XP1BR	1.0	0.58	0.42	0.80

^a Total repair sector inhibitable by aphidicolin = $1 - B/C$.

^b Excision repair sector inhibitable by aphidicolin is:

$$1 - \left(\frac{B - A}{C - A} \right)$$

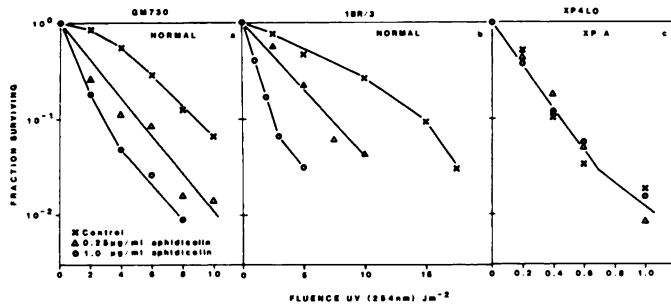


Fig. 1. Inactivation of repair proficient (a and b) and excision deficient (c) human fibroblast lines by UV (254 nm) radiation with and without postirradiation incubation with aphidicolin to inhibit the α polymerase. Dividing cell populations were washed with buffer and irradiated at cloning densities on plates at room temperature.

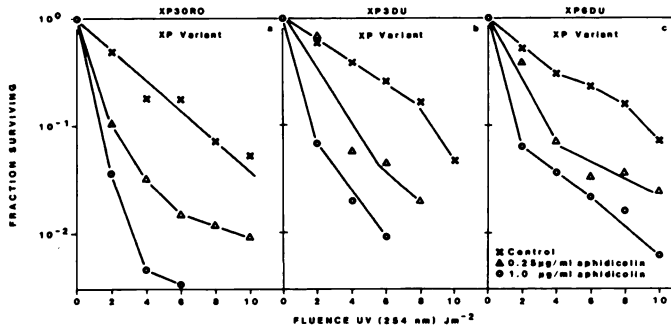


Fig. 2. Inactivation of three xeroderma pigmentosum variant lines by UV (254 nm) radiation with and without postirradiation incubation with aphidicolin. Conditions were as for Fig. 1.

to have normal excision repair activity (10). Three independently derived lines from this group are strongly sensitized to UV as a result of postirradiation incubation in aphidicolin (Fig. 2). Two other excision proficient cell lines showing slightly (Bloom's) or greatly (Cockayne's) enhanced UV sensitivity are also strongly sensitized to UV in the presence of the drug (Fig. 3, a and b). The sensitization of an XPD strain is less marked (Fig. 3c). However, it is important to note that the final UV sensitivity of XPD cells that have been incubated with 1 $\mu g/ml$ aphidicolin is similar to that of the XPA strain (Table 1).

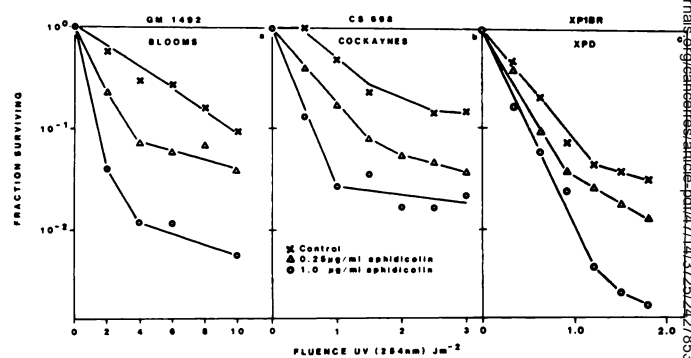


Fig. 3. Inactivation of a Bloom's (a), Cockayne's (b), and a XPD (c) cell line by UV (254 nm) radiation with and without postirradiation incubation with aphidicolin. Conditions were as for Fig. 1.

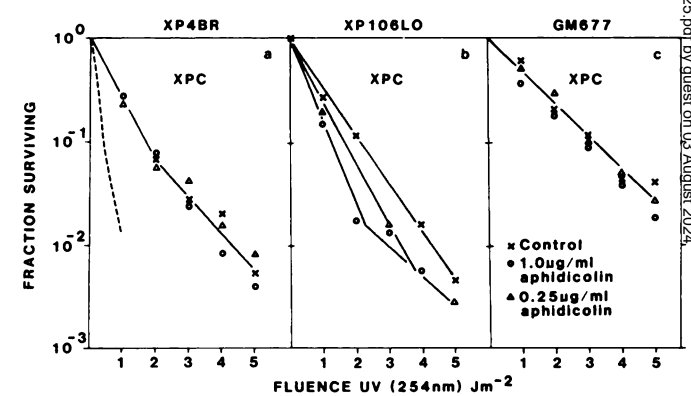


Fig. 4. Inactivation of three XPC fibroblast lines by UV (254 nm) radiation with and without postirradiation incubation with aphidicolin. Conditions were as for Fig. 1. For comparison, the survival of the XPA strain (Fig. 1c) is shown in a as - - - -.

The most interesting results arise from experiments using three XPC strains all of which have UV sensitivities intermediate between normal and totally excision deficient fibroblast lines. Despite a biochemically measurable capacity for excision repair (11), two of the XPC strains are not sensitized to UV at all by aphidicolin (Fig. 4, a and c), and a third strain (Fig. 4b) shows only an intermediate degree of sensitization.

Calculation of precise repair sectors from the type of data shown here is not possible since the irregular nature of the fluence response curves leads to continuous variations in fluence reduction factors. However, estimates can be made from the F_{10} values (fluence to reduce survival to 10%). From these values we have made two types of calculation which are included in Table 1. To begin with, we have calculated the sector of aphidicolin-inhibitable repair. This varies from zero in the XPA strain to around 90% in Bloom's and variant strains. We have then calculated the percentage of biologically effective excision repair which is aphidicolin inhibitable in each strain assuming that the difference between normal and XPA survival represents the maximum possible sector of excision repair. The three XPA strains that we have tested show a coincident inactivation response following UV (254 nm) radiation (results not shown) so that we consider the XP4LO response to be typical. The methods of calculation are shown in the footnotes to Table 1.

Rate of Aphidicolin-inhibitable Repair. By allowing intervals between the exposure to radiation and aphidicolin treatment, the kinetics of recovery from aphidicolin-inhibitable damage can be followed and this should reflect directly the rate of BEER dependent upon the α polymerase. Biochemically, there is evidence for a slow and a fast rate of excision repair of damage in human cells which comes both from studies of selective repair of essential genes (12) and from the two component kinetic curves for overall dimer excision (13, 14). We were interested to examine whether recovery of resistance to aphidicolin followed a time course similar to that for dimer excision and also whether mutants which apparently depend more heavily on the α polymerase pathway show similar rates to that seen in a normal strain. Arrested (nondividing) fibroblasts have been used in these experiments since this facilitates comparison of our results with a particularly detailed study on dimer removal in human fibroblasts (13) and also because toxicity is minimal under these conditions. A similar overall picture is seen with experiments using dividing cells (data not shown) except that UV-irradiated nondividing cells are rather more sensitive to aphidicolin (see Ref. 9). The results for a normal, a XP variant, and a Cockayne strain (Fig. 5) demonstrate that all these cell types have essentially completed biologically effective excision repair beyond the aphidicolin-sensitive step (presumably the step that involves the α polymerase) within 4 h and that there is no major difference between normal and mutant cell lines. The fast rate of excision repair of pyrimidine dimers in arrested cells (13, 14) has a half-life of 12–24 h. A more extensive description of the repair kinetics in normal

strains over a range of fluences and conditions will be described elsewhere.⁴

DISCUSSION

Details of the complex nature of excision repair in eukaryotic cells have emerged from several recent findings. For example, it has been shown that XPC cells exhibit a 3- to 4-fold enhancement of repair events at DNA attachment sites in the nuclear matrix in contrast to normal cells which apparently show a random distribution of repair events in DNA loops (15). In rodent cells, there is evidence for a preferential (faster) repair of damage to essential genes (16). Whether this is generally true for human cells is not yet clear although there is evidence for preferential repair of the active DHFR gene (12). There is also evidence for intragenomic heterogeneity of DNA repair in XPC strains (17) but this may be nonselective for active genes such as DHFR and therefore contribute little to cell survival (18). These studies and biochemical measurements (13, 14) suggest that there may be at least two components to excision repair (a slow and a fast process) although the relationship between the parameters under study is not yet clear. The results of the present study have led to the formulation of a model which also involves two components to repair. We will first discuss the basis for such a model and then its relationship to the above-mentioned studies.

Previous studies have strongly indicated that aphidicolin acts by the inhibition of excision repair dependent upon DNA polymerase α (5, 6, 8). Since postreplication repair deficient XP variant cell lines are sensitized to UV to at least as great an extent by aphidicolin treatment as normal (repair proficient) cell lines (compare Fig. 2 with Fig. 1, *a* and *b*; see Table 1), it seems unlikely that postreplication repair is an aphidicolin-sensitive pathway. The present data also indicate that excision repair may be divided into two components which are biologically effective against UV damage. Both of them are dependent on the XPA gene product (*i.e.*, an early step) but only one of them, apparently the major one under normal conditions, depends on polymerase α . The strongest evidence for two components to BEER is that while most of the fibroblast strains tested are strongly sensitized to aphidicolin (Figs. 1–3), the three XPC strains examined showed either much smaller or no sensitivity to aphidicolin (Fig. 4; Table 1) suggesting that XPC strains may be partially or even totally deficient in an aphidicolin-sensitive (polymerase α dependent) repair pathway. It is tempting to speculate that the aphidicolin-resistant repair which accounts for the difference in sensitivity between XPC and XPA strains (Table 1) could involve polymerase β . Several studies indicate that polymerase β cannot act independently in the presence of polymerase α to repair far-UV damage to human cells but is involved in a single pathway in which the enzymes act sequentially (7, 19, 20). However, in the absence of polymerase α , polymerase β clearly can have repair activity (21), so that it is possible that in XPC strains the α polymerase is altered in a way that does not effect its function in replication but does alter its ability to function in a repair complex.

Supporting evidence for aphidicolin-sensitive and -resistant pathways of repair comes from the observation that in the presence of the α polymerase inhibitor, aphidicolin, both Cockayne's and XPD cells are almost as UV sensitive as the totally excision deficient XPA strain (Fig. 1c and Fig. 3, *b* and *c*) whereas other strains (including normal, Fig. 1, *a* and *b*; variant,

⁴ S. M. Keyse and R. M. Tyrrell, manuscript in preparation.

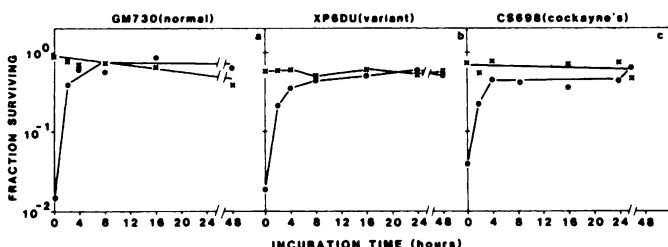


Fig. 5. Recovery from aphidicolin sensitivity as a function of incubation time following treatment with either 3 Jm^{-2} (normal and XP variant, *a* and *b*, respectively) or 1 Jm^{-2} (Cockayne's, *c*) of UV radiation at 254 nm. Nondividing cell populations were washed with buffer and irradiated at cloning densities on plates. They were incubated for the intervals indicated prior to aphidicolin exposure. Populations were treated with UV and incubated with (○) or without (×) subsequent aphidicolin treatment. Surviving fractions were corrected by the appropriate nonirradiated incubated (\pm aphidicolin) controls.

Fig. 2; and Bloom's, Fig. 3a) are sensitized to an intermediate level. This result is an indication that the Cockayne's and the XPD strains tested (as well as XPA) could be deficient in an aphidicolin-insensitive pathway. However, although reproducible results were obtained in the single strains tested, a more detailed survey is necessary to establish the generality of such a finding and to draw firm conclusions. Furthermore, it should also be noted that cockayne's cells apparently have no recognizable defect in excision repair as measured by current biochemical techniques (22).

Since the pyrimidine dimer is the major lesion induced by far-UV treatment, it is pertinent to ask whether we can relate the two components of repair that are proposed here to the fast and slow rates of excision repair of dimers observed by Kantor and Setlow (13) and Kantor and Hull (14). Recovery from UV-induced aphidicolin sensitivity is rapid in all three cell lines tested (Fig. 5) and is essentially complete within 4 h. However, even the fast component of excision repair in arrested fibroblasts occurs much more slowly than this [12–24 h; (13, 14)] so that removal of biologically effective damage dependent upon the major α polymerase-dependent pathway occurs at a rate of at least three times faster than pyrimidine dimer excision. A possible interpretation of these data is that the lethal damage is not related to pyrimidine dimers. However, a more likely explanation would appear to be that a small percentage of the dimers occurring in essential genes are repaired at a much faster rate than in the bulk DNA, and this would not have been detected in the experiments by Kantor and Setlow (13) and Kantor and Hull (14). Indeed, there is now evidence that there is a strong preferential rate of removal of pyrimidine dimers from the transcriptionally active DHFR gene compared to that in total cellular DNA (12). In this study it was shown that after 4 h incubation, many more dimers have been removed from the DHFR gene than in the genome overall, while at 24 h the levels of repair are similar. This finding appears to be entirely consistent with the fast rate of BEER we have observed in the normal and variant cell lines (Fig. 5). However, an inconsistency remains concerning the single Cockayne's cell line tested. Several lines cultured from patients with Cockayne's syndrome, including the one described herein, have been shown to be defective in recovery of RNA synthesis after UV irradiation of both dividing and nondividing cells, and it was proposed that these cells may therefore be defective in the rapid excision of a small fraction of lesions arising in actively transcribing regions (23). If the fast rate of BEER is due to preferential repair of active genes (and assuming the model for Cockayne's to be correct) then we would have predicted a much slower rate of repair in the Cockayne's strain, whereas in practice, we observed only a slightly reduced rate (Fig. 5). Again, an attempt to resolve this inconsistency will require additional experiments in several lines of Cockayne's in both dividing and nondividing cells.

In summary, we have presented evidence that BEER can be separated into at least two components, only one of which is dependent upon the aphidicolin-inhibitable α polymerase. Furthermore, the rapid rate of the α polymerase-dependent pathway relative to dimer removal is the expected biological outcome if preferential repair of essential genes were to occur. However, it remains to be seen whether the aphidicolin-independent pathway, which must account for all or a significant fraction of repair in XPC strains (but is a minor pathway in excision proficient strains), involves a modified (aphidicolin-

resistant) α polymerase or a different repair protein such as DNA polymerase β .

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