Xerodera Pigmentosum Cells with Normal Levels of Excision Repair Have a Defect in DNA Synthesis after UV-Irradiation
(postreplication repair/alkaline sucrose gradients/human fibroblasts/caffeine)

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ABSTRACT Cells cultured from most patients suffering from the sunlight-sensitive hereditary disorder xeroderma pigmentosum are defective in the ability to excise ultraviolet light (UV)-induced pyrimidine dimers from their DNA. There is, however, one class of these patients whose cells are completely normal in this excision repair process. We have found that these cells have an abnormality in the manner in which DNA is synthesized after UV-irradiation. The time taken to convert initially low molecular-weight DNA synthesized in UV-irradiated cells into high-molecular-weight DNA similar in size to that in untreated cells is much greater in these variants than in normal cells. Furthermore, this slow conversion of low to high-molecular-weight DNA is drastically inhibited by caffeine, which has no effect in normal cells. Two cell lines from classes of xeroderma pigmentosum that are defective in excision-repair show intermediate effects, with regard to both the time taken to convert newly synthesized DNA to high molecular weight and the inhibition of this process by caffeine.

Xeroderma pigmentosum (XP) is an autosomal recessive disorder, characterized by hypersensitivity of the skin to sunlight. Patients with this condition develop multiple skin lesions, culminating in skin carcinomas and early death. There are clinically two forms of the disease, the classical form and the De Sanctis–Cacchione syndrome (DSC), distinguished by the additional involvement of neurological symptoms in the latter. Cleaver (1) and many other workers have found that cells from most patients with XP (classical and DSC) are defective in the ability to remove ultraviolet (UV)-induced pyrimidine dimers from their DNA and replace them with the correct bases (reviewed in ref. 2).

Recently, complementation studies have shown that there are several genetically different forms of XP, all deficient in excision-repair (3-8). In addition, there is a further class of XP patients (termed “XP variants” in this communication) which, while exhibiting the usual clinical symptoms, seem to be completely normal in excision-repair of pyrimidine dimers (9-14). All XP lines examined have normal rates of rejoining of DNA single-strand breaks induced by ionizing radiation (14).

Despite very low levels of excision-repair in cells from most XP patients (and also in rodent cell lines), they can nevertheless tolerate the production in their DNA of over 10^6 pyrimidine dimers per cell without being killed (15). They are able to do this because they possess one or more other mechanisms for coping with pyrimidine dimers. One such mechanism is postreplication repair (reviewed in ref. 16). As in bacteria (17), newly synthesized DNA in UV-irradiated mammalian cells is much greater in cells that do not repair, than in unirradiated cells, but on prolonged incubation it eventually attains a high molecular weight similar to that from unirradiated cells (18-23). The interpretation attributed to these observations is that DNA synthesis is temporarily delayed by a pyrimidine dimer, and then continues beyond the dimer, leaving a gap that is subsequently sealed.

In this communication we show that fibroblast cultures from three XP variants have normal levels of excision-repair, but are abnormal in postreplication repair. After UV-irradiation, the time taken for the newly synthesized DNA to attain a high molecular weight similar to that in unirradiated controls is much longer than in normal cells. Furthermore, this conversion of low- to high-molecular-weight DNA is drastically inhibited by caffeine, which has very little effect in normal human cells.

MATERIALS AND METHODS

Cell Lines used in these experiments were primary fibroblasts from healthy donors and XP patients listed in Table 1.

Excision of Pyrimidine Dimers Measured by Loss of UV-Endonuclease-Susceptible Sites. This procedure has been described in detail (24). Briefly, fibroblast cells cultured as described in ref. 24 were labeled with [3H]thymidine, UV-irradiated, and incubated in the absence of radioactive label for various times. They were then mixed with an equal volume of unirradiated cells whose DNA had been labeled with [14C]-dT. DNA was extracted from the mixed cell population and incubated with or without the UV-specific endonuclease from Micrococcus luteus, which specifically nicks DNA near pyrimidine dimers (24, 25). The DNA products resulting from enzymatic attack were centrifuged through 5-20% alkaline sucrose gradients at 40,000 rpm for 135 min at 21°C in an SW56 rotor. After centrifugation, fractions were collected, radioactivity was determined, and the weight-average molecular weight of the DNA distributions was calculated. From the weight-average molecular weight the number of endonuclease-susceptible sites (presumed to be sites near pyrimidine dimers) was calculated.

Repair Replication (Insertion of Nucleotides in the Gap Left after Excision) was measured by isopycnic centrifugation of
repaired DNA in NaI gradients (26). Cells were incubated for 2 hr in the presence of 7 μM BrdU and 1 μM FdU, UV-irradiated, and then incubated in medium containing 7 μM BrdU, 10 μCi/ml of [3H]dT (2 Ci/mmol), 1 μM FdU, and 1 mM hydroxyurea for 3 hr. DNA was extracted and centrifuged to equilibrium in neutral NaI gradients, and density profiles were obtained as described (26).

Unscheduled DNA Synthesis was measured as described (3). Coverslip cultures of cells were incubated for 1 hr in the presence of [3H]dT (10 μCi/ml; 2 Ci/mmol), UV-irradiated, and incubated for a further 2 hr with the same concentration of [3H]dT. Labeling was carried out before irradiation to ensure unambiguous identification of S phase cells. Autoradiographs were prepared, and after exposure for 1 week the average number of grains per nucleus was measured in non-S phase cells.

Postreplication Repair. Cells (106) were seeded in Eagle's minimal essential medium supplemented with 15% fetal bovine serum (Flow Laboratories) in 5-cm petri dishes and incubated for 2 days. They were then washed in phosphate-buffered saline (8 g of NaCl, 0.2 g of KH2PO4, 0.2 g of KC1, 1.15 g of Na2HPO4 per liter) and exposed to an UV light fluence of 12.5 Jm−1. After a further incubation in 3 ml of warmed medium for 1 hr, they were pulse-labeled with 33 μCi/ml of [3H]dT (25 Ci/mmol). In pulse-chase experiments, the medium containing the [3H]dT was replaced after the pulse with fresh medium containing unlabeled thymidine and deoxy-
cytidine (both at 10 μM). In some experiments caffeine was present at 0.3 mg/ml throughout the whole period after irradiation. At the end of the pulse or the chase period, the cells were scraped off the petri dish with a piece of silicone rubber, into 0.3 ml of ice-cold buffered EDTA solution (0.2 g/liter of EDTA in phosphate-buffered saline). The cell suspensions were then exposed to 1.5–2 krad of γ-irradiation from a 60Co source to prevent entanglement of DNA strands on subsequent denaturation and centrifugation (21).

Alkaline sucrose gradients containing 4.7 ml of 5–20% (w/v) sucrose with 0.1 M NaOH and 0.1 M NaCl were overlaid with 0.2 ml of 2% sodium dodecyl sulfate–0.02 M EDTA. The cell suspension (0.1 ml; 1 to 2 × 106 cells) was layered into the detergent layer on the gradients, which were then centrifuged at 38,000 rpm for 70 min at 20°C in an SW50.1 rotor on a Beckman L2 or L2-65B ultracentrifuge. After centrifugation, 8-drop fractions were collected onto Whatman Grade 17 paper strips (27), and the acid-insoluble radioactivity of each fraction was measured as described (21).

RESULTS

Excision Repair in XP Variants. Fig. 1 shows results from experiments measuring various aspects of the excision-repair process in the XP variant XP30RO as compared to healthy controls. The rate of removal of UV-endonuclease-sensitive sites (pyrimidine dimers) is similar in the normal and XP30RO cells (Fig. 1a), as is the amount of unscheduled DNA synthesis (Fig. 1b) and repair replication (Fig. 1c). The latter two processes measure the amount of insertion of nucleotides in gaps left after dimer excision. The amounts of unscheduled DNA synthesis in XP7TA (results not shown) and in XP4BE (9, 10) are also similar to those in healthy controls. There remains the possibility that the final rejoicing step of excision-repair might be defective in the XP variants. This would lead to an accumulation of unrejoined breaks caused by excision of the pyrimidine dimers from the DNA after UV-irradiation. We were unable to detect any breaks either in a normal cell line (in keeping with the findings of others, e.g., see ref. 28) or in the variants, XP7TA and XP30RO, the limit of detection with our system being about 1 break/4 × 106 daltons. Thus, unlike all the other classes of XP that we have studied by these methods (24, 29, 14), excision-repair of pyrimidine dimers seems to be completely normal in the XP variants.

We have also found that the rate of rejoining of single-strand breaks induced in their DNA by γ-irradiation was the same in the XP variants XP30RO (see also ref. 14) and XP7TA, and the normal cell line C1BR (results not shown).

Postreplication Repair. Striking differences were seen between the XP variants and normal cell lines on examination of the size of DNA newly synthesized in UV-irradiated cells. Fig. 2 shows the results of a pulse and pulse-chase experiment with cells from two normal and two XP variant cell lines that were either unirradiated or irradiated with a fluence of 12.5 Jm−1. Labeling times were adjusted so that approximately equal amounts of DNA were labeled in unirradiated and irradiated cells. This eliminates labeling artifacts (21, 30, 31). The top frames show results from unirradiated cells. After a 25-min pulse, the profiles from all the cell lines were very similar: broad and in the center of the gradient. After a 75-min chase, all the DNA was near the bottom of the gradient. Because of the γ-irradiation of the cells before centrifugation (see Materials and Methods), the size of this DNA is the maximum measurable under the conditions we use. Such profiles represent

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1 The fluence rate of the UV lamp used in the experiments on postreplication repair was estimated with a calibrated thermopile. Biological and biochemical effects induced by this lamp seem, however, to be 0.5–0.7 times as great as those observed from comparable fluences from some other laboratories.
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size weight a

XP30RO breaks/10^7 (c)

are referred (bottom frames) were pulse-labeled for 60 min (solid curves), and then chased for 150 min (dashed curves). Total cpm (×10^-3): (a) 24; (b) 28; (c) 18; (d) 14; (e) 32; (f) 33; (g) 15; and (h) 10.

tween the 11th and 20th passage, and in any one experiment all the cell lines were within three passages of each other. The results from these experiments are summarized in Fig. 3. The molecular weights of the DNA from the XP variants at all times were considerably lower than those from the normal lines. We also included a classical XP cell line (XP4RO) (14) in these experiments, and the molecular weight of the DNA

a population of DNA molecules with a weight-average molecular weight of about 150 × 10^6 and will be referred to as “big DNA.” Any DNA greater than this was fragmented to this size during treatment. Without exception, profiles similar to those in Fig. 2a–d were obtained with unirradiated cells from all the cell lines studied. With cells exposed to a UV fluence of 12.5 J m^-2 (bottom frames) the profiles from pulse-labeled normal cells were very similar to those from unirradiated cells (Fig. 2e and f). With higher fluences, the DNA was smaller than in unirradiated cells (results not shown; see also ref. 23). After a 2.5-hr chase after the pulse-label, “big DNA” was obtained from normal cells (Fig. 2e and f). With UV-irradiated XP variants a quite different pattern was observed (Fig. 2g and h). After the pulse-label the DNA formed a sharp band near the top of the gradient. After a 2.5-hr chase it was in the middle of the gradient, and “big DNA” was found only after 5–8 hr. The size of the DNA from the variants in the 2.5-hr chase in the particular experiment shown in Fig. 2 was, in fact, rather greater than that seen in most of our experiments.

This behavior was highly reproducible. Profiles similar to those shown in Fig. 2e–h were obtained in 20 experiments with C1BR and two experiments each with normal lines C2BR, C3BR, C4BR, and C6RO. Results like those in Fig. 2g–h were obtained in 15 out of 16 experiments with each of the XP variants, XP7TA and XP30RO, and two experiments with XP4BE. We were concerned in these kinetic experiments that the patterns observed might be affected by the age of the cells in culture, so a series of experiments were carried out in which all the cell lines used were at approximately the same stage of their lives in culture. In these experiments the cells were be-
was intermediate between that of the normal cells and the XP variants. This was also reproducible in five experiments, and similar profiles were obtained with XP25RO (14), a DSC cell line.

Similar experiments carried out with incubation at 40° after irradiation gave very similar results. The abnormality in the XP variants is, therefore, probably not due to an unstable temperature-sensitive protein.

**Effect of Caffeine.** In rodent cell lines caffeine specifically inhibits postreplication repair, i.e., it prevents the molecular weight increase of DNA in a pulse-chase experiment in UV-irradiated cells (18, 22, 32, 33). Fig. 4 shows the effect of caffeine in the various human cell lines. Under these conditions it hardly affected the normal cell lines (Fig. 4a and b) (although a small effect could sometimes be seen with short chase times). In contrast, it almost completely prevented the molecular weight increase in the XP variants (Fig. 4e and f) during a pulse-chase. Similar results were obtained with other normal and XP variant cells. Again, with the classical (XP4RO) and DSC (XP25RO) XP lines, the inhibition was intermediate (Fig. 4e and d). Under comparable conditions caffeine caused only very small profile changes in unirradiated cells, and it did not significantly inhibit the overall rate of DNA synthesis as measured by thymidine incorporation into DNA in any of the cell lines, either irradiated or unirradiated (unpublished observations).

Thus, after a pulse-chase with a chase time of 2.5–4 hr in the presence of caffeine, all the DNA from UV-irradiated normal cells was near the bottom of the gradient, whereas that from the XP variants was near the top, and from a classical or DSC XP it was in the middle. Such an experiment, therefore, provides a very rapid diagnostic test for XP variants. In fact, a coded set containing cells from a normal, a DSC XP, and XP variant was sent from the laboratory of E. de W.-K. and D.B. to that of A.R.L., S.K.-B., and C.F.A. These were correctly diagnosed after a single “blind” experiment.

**DISCUSSION**

Cells from the majority of XP patients are partly or wholly lacking in the ability to remove pyrimidine dimers from their DNA, although there are at least four genetically distinct classes as determined by complementation tests on fused cells (3–8). These classes also differ in their levels of unscheduled DNA synthesis and repair replication (14). Cells from a further class of XP patients (XP variants) are completely normal both in excision repair of UV-induced pyrimidine dimers, measured in three different ways (Fig. 1), and in their ability to rejoin single-strand breaks after γ-irradiation. The results presented here demonstrate that these cells have a specific defect in the replication of DNA after UV-irradiation (post-replication repair). An uncharacterized deficiency in DNA synthesis occurs in UV-irradiated in “pigmented xeroderma” cells has been reported by Jung (34).

Buhl et al. showed that in one XP variant cell line (XP4BE), after UV-irradiation low molecular weight newly synthesized DNA was converted into high-molecular-weight DNA after 8 hr (23). Our more detailed kinetic study has revealed that this process was much slower in cultured fibroblasts from three XP variants [including the line used by Buhl et al. (23)] than in those from several normal subjects. After a UV fluence of 12.5 J/m² the time taken to synthesize DNA of molecular weight in excess of 150 × 10⁶ was 1.5–2.5 hr in normal cells, but took between 5 and 8 hr in the XP variants (2.5–5 hr in a classical and a DSC cell line) (Figs. 2 and 3). These differences were highly reproducible, were independent of the age of the cells in culture, and were observed with cells from three unrelated XP variant patients.

Caffeine prevented the eventual synthesis of high-molecular-weight DNA in the XP variants after UV-irradiation [as in rodent cells (18, 22, 32, 33)], while it hardly affected the process in normal cells. It thereby greatly enhanced the difference between normal cells and XP variants, thus facilitating identification of the latter (Fig. 4). Under some conditions an inhibitory effect of caffeine in the XP variants could be observed at concentrations as low as 30 μg/ml.

On the current model of DNA replication on UV-irradiated templates in mammalian cells, gaps are left in the daughter-strand DNA opposite pyrimidine dimers and are subsequently sealed by a process involving de novo synthesis (21, 35). In normal human cells, after the low UV fluence used in these experiments, the molecular weight of the DNA labeled by a pulse of [3H]thymidine in irradiated cells was similar to that in unirradiated cells. Similar results with mouse L cells irradiated with low UV fluences have been reported by Chiu and Rauth (31). This suggests that under these conditions, either synthesis took place continuously around dimers or that gaps were formed but sealed so rapidly that they could not be detected. In contrast, in the XP variants, after pulse-labeling, the DNA was much smaller in UV-irradiated cells than in unirradiated cells, suggesting that persistent gaps were formed. They were then slowly sealed during a 5–to 8-hr period, and this process, as in rodent cells, was caffeine-sensitive.

**Postreplication Repair in Excision-Deficient XP Cells.** We find the intermediate behavior of classical and DSC XP cells difficult to interpret. Two possible, but by no means proven, explanations are: (i) One or more enzymes are com-
mon to both excision repair and postreplication repair, and one of these is defective in classical or DSC cells. (ii) The pulse-chase experiments described in Results take place during the first 6 hr after irradiation. In normal excision-proficient cells some dimers will be excised during this period. Thus, fewer dimers have to be replicated in the normal cells than in the excision-deficient XP cells. This could have the effect of slowing down the conversion of low to high-molecular weight newly synthesized DNA. Such an explanation is not, of course, possible for the XP variants, which have normal excision repair. Objections can be raised to both these explanations. The effects observed were, however, reproducible and observed in two unrelated cases, and Buhl and Regan have also reported that postreplication repair was more caffeine-sensitive in a classical XP than in a normal cell line (36).

**Model to Explain Results with XP Variants.** Our results with the XP variants could be interpreted on the following speculative model. Gaps left opposite pyrimidine dimers are rapidly sealed in normal cells by an as yet uncharacterized enzyme system. We envisage that caffeine, which binds reversibly to single-stranded DNA and to UV-irradiated DNA (37), competes with these enzymes for the dimer or dimer/gap site. In normal fibroblasts these enzymes bind strongly and are, therefore, little affected by caffeine. In the XP variant cells one of these enzymes is defective and has a lower binding affinity. Gaps are, therefore, sealed more slowly, and because of the reduced binding affinity, caffeine can compete more effectively and inhibit the activity of the enzyme system. This model for the effect of caffeine is based on a similar one put forward by Harm (38) to explain its inhibition of photoreactivating enzyme in *Escherichia coli.* There are, however, many other possible explanations for our results; for example, there may be two separate postreplication repair processes, one caffeine-sensitive, the other caffeine-resistant, the latter being absent in the defective cell.

The possibility that the variants are simply lacking in polynucleotide ligase is unlikely since DNA replication in unirradiated cells, rejoining of single-strand breaks after γ-irradiation, and the rejoining step of excision repair are all completely normal (see Results). These processes are all thought to involve ligase. At this stage we do not know the nature of the defective enzyme in the XP variants. Very little is known about the mechanism and enzymology of DNA replication in undamaged mammalian cells, let alone in UV-irradiated cells. It seems fairly likely from our results, however, that a defect in postreplication repair is at least partly responsible for the clinical symptoms of xeroderma pigmentosum in these variants. This indicates that postreplication repair is a process of biological importance in human cells.

**Note Added in Proof.** Preliminary results obtained by C.F.A. have shown that the XP variants used in this work are more sensitive to UV light than normal cell lines. In addition caffeine substantially increases the sensitivity of the XP variants, whereas its effect on the survival of normal cells is very small. These results at the cellular level correlate well with the biochemical results discussed above. Also, Day (39) has found that the variant XP4BE has a decreased ability to reactive UV-irradiated adenovirus 2, as compared to several normal cell lines.

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