*EVIDENCE THAT XERODERMA PIGMENTOSUM CELLS DO NOT PERFORM THE FIRST STEP IN THE REPAIR OF ULTRAVIOLET DAMAGE TO THEIR DNA**

By R. B. Setlow, James D. Regan, James German, and W. L. Carrier

BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY, OAK RIDGE, TENNESSEE, AND NEW YORK BLOOD CENTER, NEW YORK CITY

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Abstract.—Xeroderma pigmentosum (XP) is a recessively transmitted disorder of man characterized by increased sensitivity to ultraviolet light. Homozygous, affected individuals, upon exposure to sunlight, sustain severe damage to the skin; this damage is characteristically followed by multiple basal and squamous cell carcinomas and not uncommonly by other malignant neoplasia. A tissue culture cell line was derived from the skin of a man with XP. Our measurements of ultraviolet-induced pyrimidine dimers in cellular DNA show that normal diploid human skin fibroblasts excise up to 70 per cent of the dimers in 24 hours, but that fibroblasts derived from the individual with XP excise less than 20 per cent in 48 hours. Alkaline gradient sedimentation experiments show that during the 24 hours after irradiation of normal cells a large number of single-strand breaks appear and then disappear. Such changes are not observed in XP cells. XP cells apparently fail to start the excision process because they lack the required function of an ultraviolet-specific endonuclease. These findings, plus earlier ones of Cleaver on the lack of repair replication in XP cells, raise the possibility that unexcised pyrimidine dimers can be implicated in the oncogenicity of ultraviolet radiation.

Xeroderma pigmentosum is an infrequently occurring human skin disorder which was shown more than 40 years ago[1] to be genetically determined and to follow an autosomal recessive pattern of transmission. However, the enzymatic defect has not been elucidated until now. The skin of homozygous affected individuals appears normal at birth, but usually before age 3 severe changes consequent to sun exposure appear and relentlessly progress. Freckles of various sizes and degrees of brownness appear and are accompanied by increasing dryness, telangiectasia, atrophy, and numbers of keratoses.[2] Histopathologically, there appears a combination of hyperkeratosis, marked atrophy of the dermis with irregular proliferation of certain layers, edema, dilatation of vessels, and a greatly increased accumulation of pigment.[3] The eyes are affected and photophobia may be intense. Scarring of eyelids, ulceration of cornea, and blindness may occur. The changes in the skin characteristically eventuate in some form, sometimes in multiple forms, of malignant neoplasia of the skin, and metastatic epithelioma often causes death before 30. Basal cell and squamous cell epitheliomatia in large numbers may appear over the course of years. Various other forms of benign and malignant tumors of ectodermal and mesodermal origin also occur with a much increased frequency in these affected individuals.[3] All these abnormalities appear to be the consequence of exposure to sun-

light. In addition to the skin manifestations, there may be stunting of growth and poor physical development. Genetic heterogeneity of XP, although not yet verified, is suggested by the observation that some individuals with the classic form of the disorder have normal mentality, whereas others have severe neurological abnormalities and mental deficiency in addition to the skin condition.

In the DNA of microorganisms, ultraviolet-induced cyclobutyl-pyrimidine dimers[4] are lesions: these lesions may be repaired in the dark by a process that involves excision of dimers from cellular DNA, repair replication, and rejoining of DNA strands. There are a number of reviews that summarize the relations between damage to DNA, the biological effects of the damage, and the genetics and biochemistry of repair of DNA.[5-10] However, although considerable material has been published on the clinical features and genetics of XP, there have been few studies of the basic defect in XP at the molecular level. Cleaver[11] showed that "repair replication" occurs at a reduced rate or not at all in XP cells after ultraviolet-irradiation and that the amount of unscheduled synthesis is negligible when compared with normal human fibroblasts. Although Cleaver was unable to define the enzymatic defect in XP, he suggested that the defect in man might be similar to one of the repair defects that results in increased ultraviolet-sensitivity of microorganisms. Regan, Trosko, and Carrier[12] had shown earlier that normal human cells in culture excise ultraviolet-induced primidine dimers from their DNA. These observations, combined with those of Cleaver,[11] led us to the hypothesis that defective excision of ultraviolet-induced dimers in XP is the biochemical explanation for the long-recognized severe sun sensitivity in this disorder. Our findings in support of this hypothesis have been described briefly.[13]

This report describes experiments conducted with normal human skin fibroblasts (to be referred to as control cells) and fibroblasts from the skin of a patient with the uncomplicated form of XP (to be referred to as XP cells). The data show that (1) control cells readily excise ultraviolet-induced pyrimidine dimers from their DNA, whereas XP cells do so at less than one tenth the control rate and (2) control cells, growing after ultraviolet-irradiation, exhibit an increase and then a decrease in the number of single-strand breaks in DNA (as observed in alkaline sucrose gradients), whereas XP cells do not exhibit these changes. These results are evidence that XP cells are defective in the endonuclease-mediated chain breakage that is believed[5-10] to be the initial step in dimer excision and subsequent repair of the affected DNA strand.

Materials and Methods.—Description of the patient: The patient (Ri. Wie.) presents the classical clinical picture of XP. He was born to Haitian parents who shared two ancestors, one (I.1 in the pedigree, Fig. 1) a member of the Ashkenazim and the other (I.2) of French ancestry, who had married in New Orleans early in the 19th century. At birth the patient weighed 2.5 kg and appeared normal. At age 6 months, the color of his skin began to darken, and freckles began to appear. His mother observed that he regularly began to cry a few minutes after being exposed to sunshine. At age 10–12 months he exhibited conjunctival hyperemia and evidence of photophobia; subsequently he developed extensive corneal damage necessitating removal of one eye at age 10. The first of innumerable malignant skin cancers appeared at age 10, each of which has been removed surgically. At age 12 an invasive carcinoma of the lip required extensive surgical

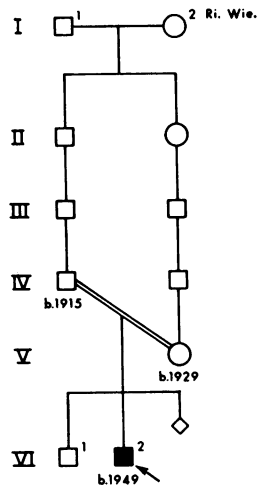

FIG. 1.—Pedigree of patient Ri. Wie. with xeroderma pigmentosum (*arrow*). He is the product of a consanguineous union (second cousins once removed).

excision, which deformed the lower part of his face. Now at age 20 he is 1.25 m tall and weighs 61 kg. He has been considered "terribly bright" by his family. He was speaking several words by age 6 months, has made unusually high grades on standardized intelligence tests, learned Braille in 6 months, and is about to enter a university.

With the use of techniques described elsewhere,[14] a fibroblast cell line named HG 418 was developed from a fragment of tissue cut from a pigmented but otherwise normal-appearing area of the patient's skin. At the time of the experiments to be described below, these XP cells had been subcultured between seven and nine times. Control cells were HS, a diploid human skin fibroblast line purchased from Microbiological Associates.

The chromosomes of cell line HG 418 were examined, at the line's fourth and seventh subculturings, with techniques described elsewhere.[14] There was no evidence of a tendency to chromosomal breakage and rearrangement, but at least one and possibly two clones with abnormal chromosomal complements were detected at the fourth subculture generation growing amid a majority of cells with an apparently normal human complement. The cytogenetic aspects of the cell line will be reported separately.[15]

Growth and treatment of cells: XP cells and control cells were grown under similar conditions in Eagle's medium with 15% fetal calf serum on ultraviolet-transparent plastic Petri dishes 5 cm in diameter (Falcon plastics).[16] Their generation times were 26 and 21 hr respectively. A typical procedure was as follows. Cells were grown at 37° to 2×10^5 per dish and were then labeled with ^{3}H-thymidine (1.9 Ci/mmole) at concentrations of 1 to 5 μCi/ml for 24 hr, at the end of which time the radioactive medium was replaced by normal growth medium. The cells were irradiated through the bottoms of the Petri dishes for 1 to 2 min with measured fluences of 265-nm radiation from a large quartz prism monochromator that was illuminated by a 500-watt, Philips capillary mercury arc. After irradiation the cells were grown for different periods of time, removed from the dishes by vigorous washing with an EDTA solution,[17] and collected by centrifugation. In Figure 2, each experimental point represents one Petri dish (3–10 $\times$ 10^4 cpm). The proportion of labeled thymine that was present as thymine-containing pyrimidine dimers was determined by acid hydrolysis of cellular components followed by paper chromatography and scintillation counting.[18] The ratio of dimers to thymine was usually measured in the fraction of whole cells that was insoluble in 5% trichloroacetic acid. In some experiments the ratio was also measured in the acid-insoluble fraction of the DNA that had been purified by Marmur's procedure[19] and in single strands of purified DNA that were greater than $\sim$30,000 mol wt. The latter material was obtained from purified DNA that was denatured in alkali, quickly neutralized, and then chromatographed on columns of Sepharose 4B (Pharmacia) to separate high-molecular-weight material from single-

stranded pieces smaller than 100 nucleotides long. The length was estimated by end-group analysis of ^{32}P-labeled DNA.

Assay for single-strand breaks: The presence of single-strand breaks in the cellular DNA was estimated by sedimentation in 5 to 20% alkaline sucrose gradients, as described by McGrath and Williams.[20] To minimize aggregation effects, only 1000–3000 cells in 0.05 ml (containing between 500 and 5000 dpm) were used. They were layered on top of 0.20 ml of 0.5 *M* NaOH that was on top of 3.6 ml of the alkaline gradient in the poly-allomer tubes of a Beckman SW-56 rotor. After 1 hr the samples were spun at 30,000 rpm for 90 min at 22°. A hole was punctured in the bottom of each tube and approximately 30 fractions were collected onto Whatman 3 MM paper disks. The disks were washed with cold 5% trichloroacetic acid and then with alcohol, dried, and counted in a toluene-BBOT (4 gm/liter) scintillator at an efficiency of 20 or 30% (depending on the scintillation counter). The recovered radioactivity was within ±15% of the input activity.

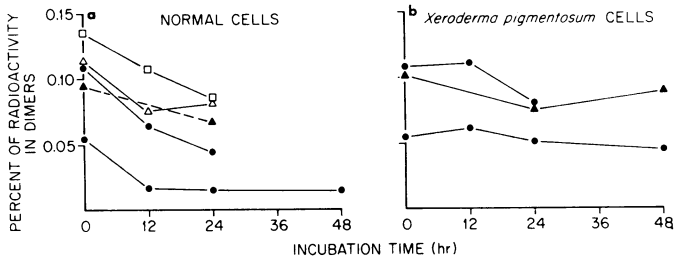

FIG. 2.—Excision of thymine-containing pyrimidine dimers from UV-irradiated, ^{3}H-thymidine-labeled, control human cells and cells from a patient with xeroderma pigmentosum. The individual curves represent separate experiments with different UV exposures at 265 nm (the lowest curves 75 erg/mm^2 and the upper ones about 150 erg/mm^2) and different methods of measuring excision. The solid points are dimers in the acid-insoluble fraction of the cells, and the open ones are from experiments in which the DNA was purified before hydrolysis and analysis for dimers (see *Methods*).

Results and Discussion.—Excision of dimers: Figure 2 shows the results of several experiments to determine the amount of excision of ultraviolet-induced pyrimidine dimers from the DNA of irradiated control and XP cells. The results were similar for dimers obtained from the hydrolysates of acid-insoluble fractions of whole cells, from the DNA purified by Marmur's procedure, and from single strands greater than ~100 nucleotides long that had been isolated from the latter DNA (see *Methods*). The control cells excised dimers in amounts comparable to the values previously reported for other types of human cells.[12] After irradiation by 150 erg/mm^2, 50–60 per cent of the dimers were removed in 24 hours with little or no additional excision thereafter. With a lower ultraviolet dose a greater proportion of dimers—up to 70 per cent—was excised by 12 hours. The absolute numbers of dimers excised per hour were the same at the two doses.

In striking contrast to the results for control cells, XP cells exhibited little or no excision. The average initial excision rate in XP cells is at least ten-fold smaller than that observed in control cells. Any slight turnover of radioactivity due to DNA breakdown of the DNA of XP cells could account for the small amount of apparent excision sometimes observed in the XP cells.[9]

Strand breaks in DNA: Figure 3 shows typical results of centrifugation in

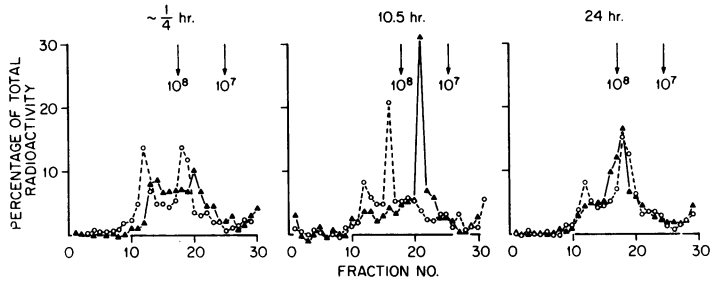

FIG. 3.—Normal cells. Typical sedimentation patterns (90 min; 30,000 rpm; SW-56 rotor) in alkaline sucrose gradients of the DNA of cells layered on top of the gradients. Cells were unirradiated (—○—) or irradiated at zero time with 150 ergs/mm² of 265 nm (—▲—) and grown for the indicated times before layering on gradients. Cells per gradient, ~2000; cpm per gradient, 150–300; counting time per point, ≥20 min. The arrows indicate the expected positions of single-stranded molecular weights of 10^7 and 10^8 as estimated from separate sedimentation experiments of ^{3}H-thymidine-labeled T4 phage DNA in alkali and Studier's[21] relation between sedimentation constant and molecular weight.

alkaline sucrose gradients to estimate changes in the single-stranded molecular weight of the DNA of control cells after ultraviolet-irradiation. The distribution of sedimentation coefficients of the DNA of unirradiated cells is somewhat variable, as indicated by the control samples in Figure 3. We do not know the reasons for the variability nor do we find it pertinent here to attempt explanation of the shapes of the distribution curve. The conclusions we reach are independent of these uncertainties.

Ten hours after irradiation the sedimentation constant of the DNA of irradiated cells is obviously smaller than that of unirradiated material, but by 24 hours the irradiated sample appears almost the same as the control.

The sequence of steps in repair is thought to be:[5–10] (1) a single-strand break on one side of a dimer; (2) dimer excision as a result of a second break on the same strand; (3) repair replication which fills in the resulting gap; and (4) closure of the gap by ligase action.

The decrease in molecular weight in irradiated control cells is interpreted as arising from step 1 events that have not yet progressed to step 4. When the repair process is completed, the single-strand breaks disappear. The average separation between dimers may be computed as follows. At the dose used ~0.12 per cent of the thymine is in pyrimidine dimers. If we take thymine as 0.3 of the DNA bases and use the observation that the numbers of TT, CT, and CC dimers are in the ratios 6:3:1,[22] we calculate the average distance between dimers in a single strand to be 1.4×10^6 daltons.[23] The latter number is very much smaller than the sizes of the pieces observed from unirradiated cells, $\sim 10^8$ daltons. At 10 hours, by which time excision of ~30 per cent of the dimers has made a gap every 5×10^6 daltons, a peak molecular weight of $\sim 4 \times 10^7$ is observed. Therefore, even at this time most of the breaks and gaps have already been closed. The time scale for excision and gap closing, relative to the cell cycle time, is about the same as is observed for strains of *E. coli* that are able to excise dimers.[24]

The change in molecular weight readily demonstrable in control cells, was not observed under similar conditions with XP cells (Fig. 4). The sedimentation coefficients of DNA from irradiated cells are, if anything, greater than those from unirradiated ones. (Again we see variability in the control patterns; some of this variability seems to be associated with the amount of labeling of the cells, in that the molecular weight appears smaller in the more highly labeled cells.) A simple interpretation of this result is that XP cells do not possess a mechanism for producing DNA chain breaks near a dimer, the first step in excision, so that, as actually shown in Figure 2*b*, the dimers are not excised.

Conclusions.—A reasonable and direct explanation for our results from these two complementary experiments is that XP cells are deficient in a functional form of an ultraviolet-specific endonuclease essential to initiation of repair. Clea-

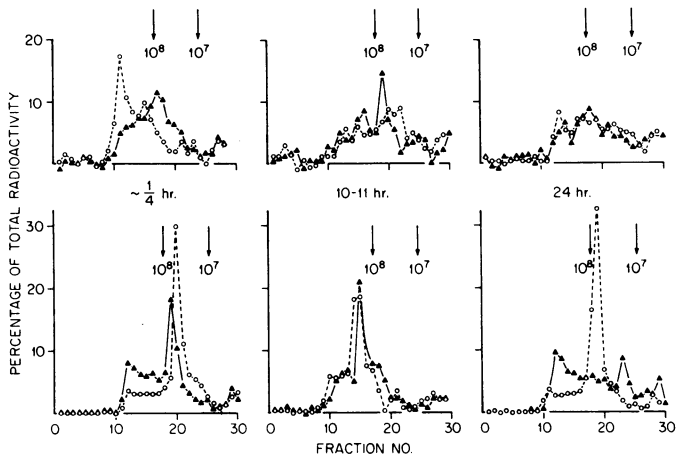

FIG. 4.—Xeroderma pigmentosum cells. Typical sedimentation patterns in alkaline sucrose gradients of the DNA of cells layered on the top of the gradients. Procedure the same as in Fig. 3 except that the lower quarter-hour and 24-hr samples had fivefold more label per cell. —▲— irradiated; —○— unirradiated.

ver[25] obtained data on the presence of repair replication in XP cells exposed to X rays or, after they had incorporated bromodeoxyuridine, to light. From these less direct experiments he reached a conclusion similar to ours. Other indirect mechanisms which might interfere with the proper functioning of an otherwise normal enzyme have not been ruled out, however. In any event, excision of dimers in XP cells does not take place because the first step does not occur.

Even if XP cells are truly deficient in the dimer-specific endonuclease, the question still remains as to whether this is the primary enzymatic defect in the disease. Whether or not the endonuclease deficiency is the basic defect, it is possible that the accumulation of pyrimidine dimers and other nonexcised products in the DNA of a skin cell either directly or indirectly causes or predisposes to neoplastic transformation of that cell. This is the first time that pyrimidine dimers *per se* have been experimentally implicated in the process of carcinogenesis.

Both the biochemical observations of dimer accumulation in the DNA of XP cells, which are predisposed to neoplasia, and the cytogenetic detection (to be reported elsewhere[15]) of abnormal stemlines in the cell cultures indicate the potential importance of their study in relation to the problem of neoplasia.

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[16] These Petri dishes were kindly supplied by Dr. William Chandler of Falcon Plastics. The transmission is 25% at 265 nm.

[17] The solution contained, per liter of water: NaCl, 8.0 gm; KH_2PO_4, 0.2 gm; KCl, 0.2 gm; Na_2HPO_4, 1.15 gm; disodium ethylenediamine tetraacetate, 0.2 gm.

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[23] For a DNA-labeled in thymine, TT will have twice the activity of CT and CC will be unlabeled. The numbers of dimers per nucleotide will be TT, 1.44×10^{-4}; CT, 0.72×10^{-4}; and CC, 0.24×10^{-4}. The total dimers per nucleotide will be 2.4×10^{-4}, and the average number of nucleotides between dimers will be 0.42×10^{4}. If the molecular weight per nucleotide is 325, the average molecular weight between dimers is 1.4×10^{6}.

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