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CHANGES INDUCED BY ULTRAVIOLET LIGHT IN THE SUPERHELICAL DNA OF LYMPHOCYTES FROM SUBJECTS WITH XERODERMA PIGMENTOSUM AND NORMAL CONTROLS

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SUMMARY

Patients with the light-sensitive disease xeroderma pigmentosum (XP) are genetically heterogeneous. Most patients are defective in the excision repair of u.v.-induced DNA damage while some, the XP-variants, seem proficient in excision repair but replicate u.v.-damaged DNA with difficulty. The former have so far been assigned to 5 complementation groups (A–E) of which B and E are extremely rare.

Information on the nature of the defect in XP is contradictory. Studies based on the sedimentation of DNA in alkaline gradients suggest that patients of complementation group A can perform the first step of excision repair ('incision') while others, based on the elution by alkali of the DNA of cells lysed on filters, suggest that they cannot. We have therefore investigated the repair of DNA damaged by u.v. irradiation using a method based on a precisely defined theoretical approach. A single break in one of the strands of a double-stranded and supercoiled circle of DNA releases supercoiling and reduces its rate of sedimentation in sucrose gradients. Structures resembling nuclei ('nucleoids') containing superhelical DNA may be obtained from human lymphocytes and their rate of sedimentation is influenced by the integrity of their DNA.

Analysis of the sedimentation of nucleoids from normal and XP lymphocytes in sucrose gradients containing different concentrations of ethidium indicates that their DNA is similarly supercoiled. The lymphocytes of patients of complementation groups A, C and D repair damage induced by γ-rays just like controls. However, they cannot repair normally DNA damaged by u.v. irradiation; the cells are defective in the first step of excision repair.

The XP-variant and XP-heterozygous cells repaired u.v.- and γ-ray-induced damage like controls.

Some problems regarding the nature of the defect in XP are discussed.

INTRODUCTION

Xeroderma pigmentosum (XP) is an autosomal recessive, light-sensitive disease caused by at least six distinct mutations. Patients homozygous for 5 of these mutations are defective in the excision repair of photolesions (Cleaver, 1968; de Weerd-Kastelein, Keijzer & Bootsma, 1972, 1974*a*, *b*; Kraemer *et al.* 1975), while the others (XP-variants) show a defect in the replication of u.v.-damaged DNA which can be enhanced by caffeine (Lehmann *et al.* 1975).

Excision repair is a complex process involving the incision of the damaged DNA strand, removal of the principal photolesion (pyrimidine dimers), synthesis of a new patch of DNA complementary to the undamaged strand and finally ligation of this segment to the existing strand (Cleaver, 1974*a*). It is generally thought that in XP cells an early stage in the process is impaired since patients with 2 distinct mutations (i.e. those of complementation groups A and C) are defective in dimer excision (Paterson, Lohman & Sluyter, 1973), as are other patients whose complementation group is undetermined (Setlow, Regan, German & Carrier, 1969; Cleaver & Trosko, 1970). However, it has proved difficult to test whether XP cells are defective in the incision step of excision repair because the standard techniques of sedimentation in alkaline sucrose gradients seem too insensitive to detect the small number of incisions made during repair (Setlow *et al.* 1969; Lohman, Bootsma & Hey, 1972; Huang, Kremer, Laszlo & Setlow, 1972; Kleijer, Hoeksema, Sluyter & Bootsma, 1973). Nevertheless, Cleaver (1974*b*), using a modified procedure was able to detect u.v.-induced changes in the sedimentation coefficient of DNA and he concluded that XP cells from patients with severe neurological abnormalities (De-Sanctis-Cacchione's syndrome) either were able to incise u.v.-damaged DNA and accumulated breaks as a result of a post-incision block, or degraded their DNA non-specifically. More recently, Dingman & Kakunaga (1976), using a different sedimentation technique, concluded that normal, XP-variant and XP cells of complementation group A incise u.v.-damaged DNA at the same rate. The last named, however, failed to rejoin the resulting breaks, while the XP-variant rejoined them slowly. In contrast, Fornace, Kohn & Kann (1976) have claimed that the pattern of elution of DNA by alkali from cells lysed on filters indicates that XP fibroblasts of complementation groups A, B, C and D do not incise u.v.-damaged DNA, while XP variant cells incise normally but reseal the breaks at a slow rate. In view of these discrepancies, it seemed to us useful to study the incision and rejoining of u.v.-irradiated DNA in normal and XP cells by a method based on a precisely defined theoretical approach. This is as follows.

One single-strand break in a double-stranded and circular DNA releases any supercoiling from that DNA. As supercoiled DNA is compact, it sediments in sucrose gradients more rapidly than the relaxed and so extended form. The rate of sedimentation of DNA is therefore a sensitive index of the integrity of a DNA molecule (Bauer & Vinograd, 1971). Structures resembling nuclei and containing superhelical DNA may be obtained by lysing cells in non-ionic detergents and high salt concentrations. These 'nucleoids' contain nearly all the nuclear RNA and DNA but are depleted of protein (Cook, Brazell & Jost, 1976). As their DNA is supercoiled the nucleoids sediment in sucrose gradients more rapidly than their γ-irradiated counterparts which contain broken and extended DNA. Repair of the breaks restores the DNA to its original conformation and re-establishes the normal sedimentation rate. As even only one single-strand break in a very long stretch of nucleoid DNA releases all supercoiling from that stretch, very few breaks in nuclear DNA may be readily detected (Cook & Brazell, 1975, 1976*b*).

MATERIALS AND METHODS

Fibroblast strains from 4 patients with XP (H.10785/PRU 8097; H.13235/PRU 10903; H.10698/PRU 8161; H.13498/PRU 11237), the father of the first patient (H.10787/PRU 8097) and 2 normal controls (H.9172 and H.9527) were obtained from skin biopsies cultured by a modification of the technique of Hsu & Kellog (1960).

DNA repair synthesis following u.v. irradiation (mainly 254 nm) was studied by autoradiography (Giannelli & Pawsey, 1974), and the maturation of DNA chains synthesized after u.v. irradiation by the procedure of Lehmann *et al.* (1975). XP cell strains were assigned to the appropriate complementation group by analysing their u.v.-induced DNA repair synthesis in heterokaryons produced with the help of inactivated Sendai virus (Harris & Watkins, 1965) as described by Giannelli & Pawsey (1976). Reference XP cell strains of known complementation groups were obtained from the American Type Culture Collection, Rockville, Md. and the Institute for Medical Research, Camden, New Jersey.

Lymphocytes were isolated and their ability to repair γ-ray and ultraviolet irradiation damage was monitored as described by Cook & Brazell (1976*b*). Usually, white cells were isolated from blood samples collected in London, suspended in RPMI 1640 medium containing 10% serum and sent on ice to Oxford for analysis. Experiments were completed within 24 h of collecting the samples. Comparative experiments were conducted blind and experimental samples were always spun at the same time in the same rotor as the control samples.

RESULTS

Fibroblasts of 3 patients (H.10785/PRU 8097; H.13235/PRU 10903 and H.10698/PRU 8161) but not those of a fourth (H.13498/PRU 11237) are defective in unscheduled DNA synthesis. The XP heterozygous strain reaches a normal level of unscheduled DNA synthesis after small doses of u.v. light but shows a statistically significant deficit after high doses (Table 1). Cell fusion studies indicate that the unscheduled DNA synthesis of cell strains from each of the 3 excision-defective patients improves markedly in heterokaryons made with 2 of the reference strains, but not with the third. On the basis of these findings, our patients can, therefore, be assigned to complementation groups A, C and D respectively (Table 2). On the other hand, the fibroblasts from the fourth patient respond to u.v. irradiation as expected of an XP-variant, because they show a u.v.-dependent delay in the maturation of new DNA chains, which can be enhanced by caffeine (Fig. 1).

The production of DNA damage and its repair in the white blood cells of patients and controls was monitored by comparing the distance sedimented by unirradiated and irradiated nucleoids in a sucrose gradient lacking ethidium. As factors other than DNA integrity (e.g. the degree of supercoiling) might influence the behaviour of nucleoids, we also compared the sedimentation of unirradiated nucleoids from patients and controls in gradients containing ethidium (Fig. 2). As the concentration of ethidium in these gradients is increased, the distance sedimented by the nucleoids falls to a minimum and then rises again – as expected of superhelical DNA (Bauer & Vinograd, 1971). Since the nucleoids from untreated XP and control cells behaved identically, we concluded that the integrity and degree of supercoiling of their DNA is similar. (The data on normal individuals included experiments accumulated over a 2-year period and confirm the initial observation of Cook & Brazell (1976*b*)).

Repair of the damage caused by γ-irradiation was studied by irradiating white blood cells (9·6 J kg^{-1}) and then incubating them for different periods. The cells were then

Table 1. *Level of unscheduled DNA synthesis induced by u.v. irradiation in the experimental fibroblast strains**

Patient code	Diagnosis	u.v. dose, J m^{-2}	Unscheduled DNA synthesis % of controls	Significant departures from normality†
H 10785/PRU 8097	XP (with neurological complications)	10	12·0	+
		50	10·8	+
		100	10·5	+
		150	11·9	+
H 13235/PRU 10903	XP	50	18·0	+
		100	12·0	+
		150	14·0	+
H 10698/PRU 8161	XP	10	14·3	+
		50	13·3	+
		100	11·5	+
		150	9·0	+
H 13498/PRU 11237	XP	10	96	−
		150	104	−
H 10787/PRU 8097	XP heterozygote	10	98	−
		50	73	+
		100	73	+

* Two samples of approximately 100 cells were used to measure the unscheduled DNA synthesis which took place in the 75 min following each u.v. dose.

† +, Differences statistically significant at the 0·1 % level; −, differences statistically not significant at the 5 % level.

Table 2. *Percentage increase of unscheduled DNA synthesis over the nuclei of test fibroblasts after fusion with reference XP cell strains**

Complementation group of reference strain	Test XP fibroblast strains		
	H 10785/PRU 8097	H 13235/PRU 10903	H 10698/PRU 8161
A	−6†	302	259
C	577	26†	266
D	645	559	−2†

* A minimum of 50 heterodikaryons containing nuclei not undergoing DNA replication and with different sex markers were analysed in each cross. These were exposed to u.v. light (100 J m^{-2}) 24 h after fusion and the cells were allowed to undergo unscheduled DNA synthesis for 1·5 h prior to harvesting.

† Statistically insignificant decreases or increases in unscheduled DNA synthesis indicating that the 3 experimental XP cell strains are not complemented by strains of group A, C and D respectively.

applied to gradients lacking ethidium and the sedimentation rate of the nucleoids measured. γ-irradiated nucleoids sediment at about one third the rate of their unirradiated counterparts; the radiation breaks DNA, releasing supercoiling and so extending the DNA. Incubating irradiated cells at 37 °C increases their sedimentation rate as the DNA is repaired, so that after 5 h the rate is restored almost to that of unirradiated controls (Fig. 3). (Again the data accumulated on normal individuals

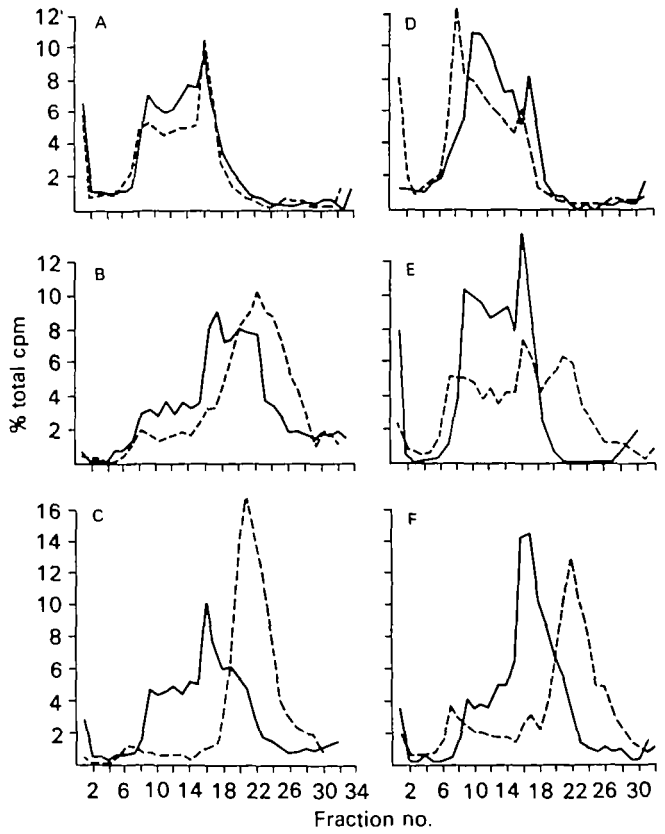

Fig. 1. Maturation of DNA chains synthesized after u.v. irradiation. Cells irradiated or sham-irradiated with u.v. light (mainly of 254 nm) at a fluence of 12·5 J m^{-2} were incubated in normal medium or in medium containing 0·3 mg/ml of caffeine for 1 h. [^{3}H]thymidine (10 μCi/ml; 23 Ci/mmol) was then added to the medium for a 1-h pulse. The cells were then harvested or incubated in isotope-free medium containing thymidine and deoxycytidine (10 μM) for a 2·5-h chase. After harvesting the cells were exposed to 15–20 J kg^{-1} of γ-irradiation from a Co^{60} source, immediately lysed on top of alkaline sucrose gradients (4·4 ml, 5–20 %) and spun for 75 min at 40000 rev/min in a SW 56 rotor of a Beckman L2-65B ultracentrifuge. Fractions were collected from the bottom of the tube on paper strips as described by Carrier & Setlow (1971) – sedimentation was towards the left. The behaviour of [^{3}H]thymidine-labelled DNA from the control (●——●) and the XP fibroblast strain no. H.13498/PRU 11237 (●- - - -●) is compared after the pulse (A, B, C) and the 2·5-h chase (D, E, F). A, D, unirradiated cells; B, E, irradiated cells; C, F, irradiated cells treated with caffeine. Note that u.v. light affects the maturation of DNA chains to a greater extent in the XP cells than in controls and that this effect is potentiated by caffeine.

confirm the initial results of Cook & Brazell (1976*b*).) No differences in the rates of repair of γ-ray induced damage were detected in any of the samples of the patients' cells.

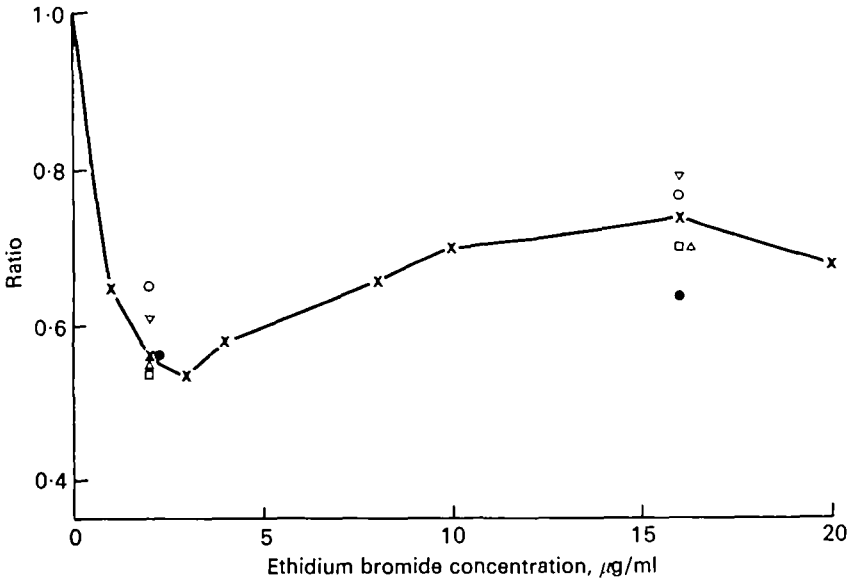

Fig. 2. The effect of ethidium on the sedimentation of nucleoids. The distance sedimented by nucleoids in sucrose gradients containing different concentrations of ethidium is expressed as a ratio relative to that of unirradiated nucleoids sedimenting in the absence of ethidium. Nucleoids were obtained from controls (×); from patients with xeroderma pigmentosum complementation group A (△), C (□) and D (○); from a patient with xeroderma pigmentosum variant form (▽); and from the father of a xeroderma pigmentosum patient, complementation group A (●). For the controls, the standard errors of the mean at each point were less than 0·01.

In contrast to γ-rays, low doses of u.v.-light do not break the DNA, but incision of DNA strands should occur subsequently, in the course of excision repair, during the incubation of the irradiated cells. It can be expected that incision would slow the sedimentation of nucleoids as DNA supercoils are removed and, as repair is completed, ligation should restore the sedimentation to the fast rate characteristic of nucleoids from unirradiated cells. The data summarized in Fig. 4(*a*) show that this is the case, confirming earlier results (Cook & Brazell, 1976*b*).

On the other hand, the cells from patients with xeroderma pigmentosum (complementation groups A, C and D) do not conform to this pattern. At all times following u.v. irradiation the nucleoids from these cells sediment rapidly in the absence of ethidium (Fig. 4B, C, D): the cells of these patients are thus unable to 'incise' normally, so that the nucleoids retain their supercoiling. In contrast, cells from the XP-variant and the XP-heterozygote showed marked changes in nucleoid sedimentation under these conditions, and, in this respect, they did not appear to differ significantly from the controls (Fig. 4E, F): thus they are capable of normal levels of incision with consequent release of supercoiling.

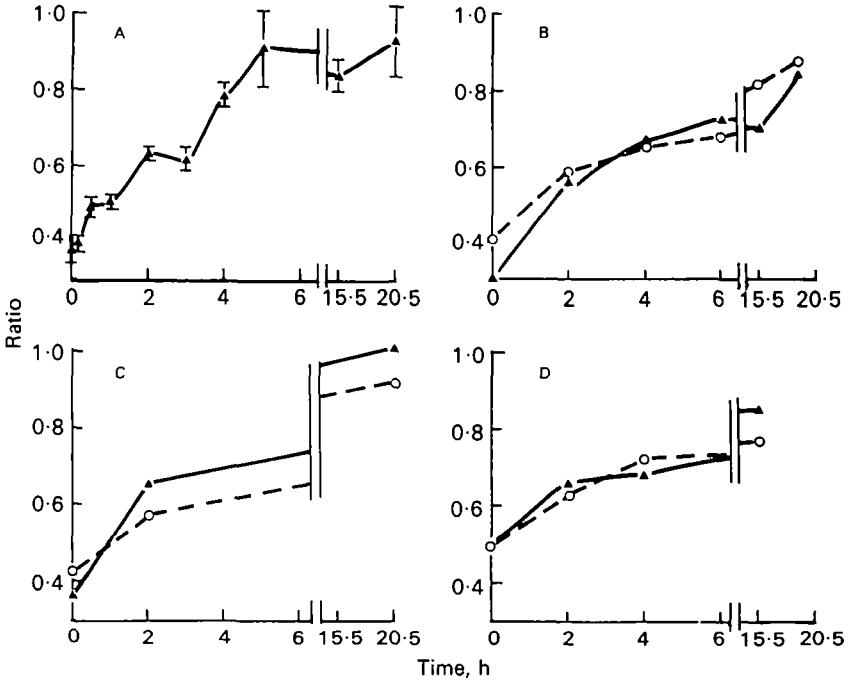

Fig. 3. Repair of γ-ray induced damage. White blood cells were γ-irradiated (9·6 J kg^{-1}) and incubated for different periods at 37 °C before the rate of sedimentation of their nucleoids was determined. The distance sedimented by nucleoids from irradiated cells in sucrose gradients lacking ethidium is expressed as a ratio relative to that of nucleoids from unirradiated cells which had been treated similarly. A, control cells (▲). The means of the ratios were obtained from at least 8 different blood samples determined on different occasions. Error bars give the standard error of the mean. Each of the remaining panels (B–D) summarizes the results of the repair of γ-ray-induced damage in 2 samples of white cells obtained at the same time, one from a control (▲), the other from a patient (○– – –○). The patients were XP complementation groups C (B), D (C), and XP variant (D).

DISCUSSION

Complementation data on 3 series of excision-defective XP indicate that more than 90% of such patients belong to complementation groups A, C and D (de Weerd-Kastelein *et al.* 1974*b*; Kraemer *et al.* 1975; Giannelli *et al.*, in preparation). We have analysed representatives of these 3 major complementation groups and have found evidence of abnormalities in the changes in DNA integrity that normally accompany u.v. irradiation. In particular, the rate of sedimentation of nucleoids from irradiated XP cells remained high, indicating that the cells were incapable of normal incision. This is in keeping with the observations of Fornace *et al.* (1976) on XP fibroblast strains of complementation groups A, B, C and D and supports the idea of an endonuclease-related defect in this type of XP. The precise nature of the enzymic defect in the disease is, however, still unknown.

The apparent discrepancy between the genetic heterogeneity and biochemical homogeneity of excision-defective XP may be explained by 2 alternative hypotheses.

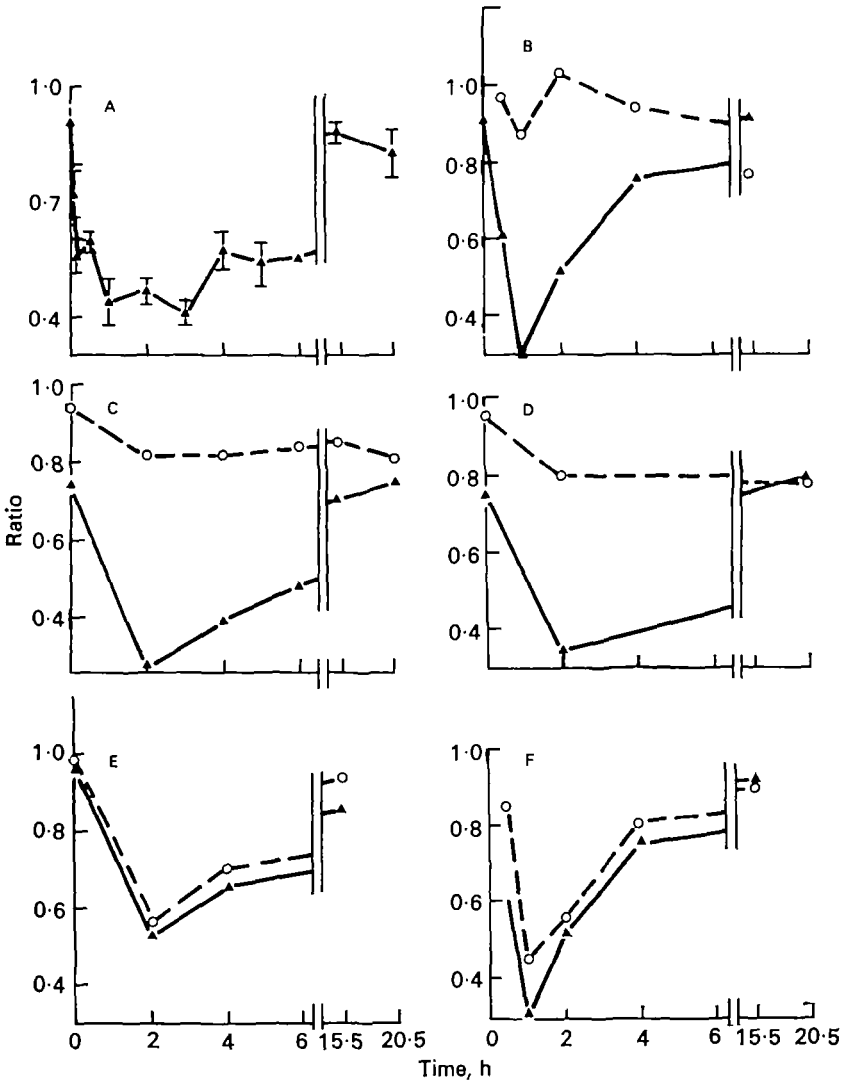

Fig. 4. Repair of u.v.-induced damage. White blood cells were irradiated with ultraviolet light (2·5 J m^{-2}; 0·5 J m^{-2} s^{-1}) and incubated for different periods before the rate of sedimentation of their nucleoids was determined. The distance sedimented by nucleoids from irradiated cells in sucrose gradients lacking ethidium is expressed as a ratio relative to that of nucleoids from unirradiated cells which had been treated similarly. A (▲), the means of the ratios were obtained from at least 12 different blood samples from 8 people determined on different occasions. Error bars give the standard error of the mean. Each of the remaining panels (B–F) summarizes the results of the repair of u.v.-induced damage in 2 samples of white cells obtained at the same time, one from a control (▲), the other from a patient (○– – –○). The patients were XP complementation group A (B), C (C), D (D), XP variant form (E) and the father of an XP patient of complementation group A (F).

The various loci may all be independently involved in the production and control of the factor responsible for the first step of 'dimer excision repair'; or, such repair may be under the control of an enzyme complex functioning in a coordinated way, so that a defect in any of its components would cause inactivation of the entire complex (Haynes, 1966; Kleijer *et al.* 1973). Tanaka, Sekiguchi & Okada (1975) have reported however, that if the u.v. endonuclease specific to phage *T4*, is introduced into XP cells with the help of Sendai virus, unscheduled DNA synthesis is restored. Cook, K., Friedberg & Cleaver (1975) have shown that cell-free extracts of XP fibroblasts of complementation groups A, C, D and E can excise pyrimidine dimers from u.v.-irradiated *Escherichia coli* DNA treated with the same phage endonuclease. However, recent experiments show that extracts from cells of complementation groups A, C and D are able to excise pyrimidine dimers from purified DNA even in the absence of the phage endonuclease. On the other hand, extracts of cells from one of these three complementation groups (i.e. group A) could not excise dimers from u.v.-irradiated chromatin (Mortelmans *et al.* 1976).

These conflicting results suggest that the genetic heterogeneity of XP cannot be simply explained. Moreover, studies on the kinetics of complementation of different XP cell strains indicate that the 'XP-factor' is complex, and might involve the products of at least 2 genes (Giannelli & Pawsey, 1974, 1976, and unpublished results).

XP and XP-variant are clinically virtually indistinguishable, but they appear to be due to distinct metabolic defects. In keeping with this, our results indicate that XP-variant lymphocytes are proficient in the excision repair of u.v.-induced DNA damage; but they fail to show – at least in the absence of caffeine and at low u.v. doses – the delay in rejoining of incision breaks observed by Fornace *et al.* (1976) and Dingman & Kakunaga (1976) in fibroblast strains from 3 other patients.

Finally, 2 attempts to detect the heterozygous state of the father of our first patient by the 'nucleoid technique' were unsuccessful. Heterozygosity tests in XP can, however, be performed by the procedure described by Giannelli & Pawsey (1974).

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