

# Xeroderma pigmentosum neurological abnormalities correlate with colony-forming ability after ultraviolet radiation

(DNA repair/premature aging/carcinogenesis/acute sun sensitivity/genodermatosis)

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Communicated by Victor A. McKusick, January 12, 1978

**ABSTRACT** Xeroderma pigmentosum is an autosomal recessive disease in which DNA repair processes are defective. All xeroderma pigmentosum patients develop premature aging of sun-exposed skin, and some develop neurological abnormalities due to premature death of nerve cells. Sensitivity to ultraviolet radiation of 24 xeroderma pigmentosum fibroblast strains was studied *in vitro* by measuring each strain's ability to divide and form colonies after irradiation. The most sensitive strains were derived from patients who had an early onset of neurological abnormalities; less sensitive strains were from patients with a later onset; and the most resistant strains were from patients without neurological abnormalities. The UV sensitivities of strains from each member of a sibling pair with xeroderma pigmentosum were identical, indicating that UV sensitivity of xeroderma pigmentosum strains is determined by the patient's inherited DNA repair defect. The results suggest that effective DNA repair is required to maintain the functional integrity of the human nervous system by preventing premature death of neurons.

Cells from patients with xeroderma pigmentosum (XP), an autosomal recessive disease, have abnormal repair of DNA that has been damaged by ultraviolet (UV) radiation or certain chemical carcinogens (1). There are currently six known genetic forms of XP: the variant form, with a normal rate of UV-induced unscheduled DNA repair synthesis (a measure of excision repair) but abnormal postreplication repair (2), and five excision repair-deficient forms, which have been classified into complementation groups A-E by cell fusion of their strains (3). All XP patients develop excessive UV damage in sunlight-exposed areas at an early age. Some XP patients also develop neurological abnormalities due to the premature death of central nervous system neurons in the absence of recognizable and specific histopathology (1). We have, therefore, considered the neurological abnormalities of XP to be the result of an abnormal aging of the human nervous system (1).

Post-UV colony-forming ability of human fibroblasts *in vitro* is markedly affected by the ability of the cells to repair their UV-damaged DNA to the functional state required for cell survival and division (4, 5). In this paper we present in detail our findings concerning the relationship of the neurological abnormalities of XP to the post-UV CFA of 24 XP fibroblast strains.

## MATERIALS AND METHODS

**Fibroblast Strains.** Eighteen of the 24 XP strains and all five of the normal control donor strains studied were from the American Type Culture Collection, Rockville, MD. Fourteen of the XP strains were from patients of the original National Institutes of Health series (1) or additions thereto. Strains

XP2NE and XP3NE (6, 7), XP8LO (7), XP4LO, and XP26RO (8) were gifts from Dirk Bootsma (Erasmus University, Rotterdam). Strain XP6TO (9) was a gift from Hiraku Takebe (Kyoto University, Kyoto). All XP strains are identified by the international nomenclature for XP strains (8). Fibroblast stock cultures were grown without antibiotics in Ham's F12 medium modified according to Coon and Weiss (10) but with 0.518 g of  $MgSO_4 \cdot 7H_2O$  per liter, supplemented with 10% fetal calf serum. They were incubated at 37° in an atmosphere of 5%  $CO_2$ /95% air with more than 95% humidity. Complementation group assignments were determined by cell fusion studies as previously reported (1, 3, 7, 9, 11) or by unpublished fusion studies in this laboratory (in the cases of the XP17BE and XPKABE strains used in this work). Rates of UV-induced unscheduled DNA synthesis were determined in this laboratory by methods previously described (1, 11).

**Post-UV Colony-Forming Ability.** Petri dishes (100 mm) containing Ham's F12 medium, modified as above but supplemented with 20% fetal calf serum, were each inoculated with approximately  $10^5$  cells. While the cells were still in logarithmic phase 5-7 days later, they were washed with  $Ca^{2+}$ -free and  $Mg^{2+}$ -free Hanks' balanced salt solution, then flooded with 5 ml of this solution, and irradiated in open dishes at room temperature with 254-nm UV light from a germicidal lamp (General Electric lamp no. G15T8) at an incident flux of 0.67-0.86 erg/mm<sup>2</sup> per sec. The flux was determined both by the malachite green leukocyanide assay (12) and by a factory-calibrated IL770 radiometer (International Light, Newburyport, MA), which agreed with the results of the leukocyanide assay to two significant figures. Immediately after irradiation the cells were trypsinized and replated into new dishes at concentrations appropriate to the strain's UV sensitivity and to the dose of UV. Cells were fed after one week and biweekly thereafter. After 14-21 days the cells were fixed with methanol/acetic acid (3:1, vol/vol) and then stained in the dishes with trypan blue. The dishes were examined for colonies (aggregates of 30 or more cells) by means of a dissecting microscope. At every UV dose in each experiment, including zero, two to four groups of dishes were inoculated, each group containing four to eight dishes and differing from the other groups by half-log increments in the number of cells plated per dish. At each dose, one to three of these groups contained colonies at a sufficient density and with a sufficiently low background (i.e., non-colony-forming cells) to be readily scored. The colony-forming efficiency, defined as the number of colonies obtained divided by the number of cells plated, was determined for each dose

Abbreviations: XP, xeroderma pigmentosum; UV, ultraviolet; CFA, colony-forming ability.

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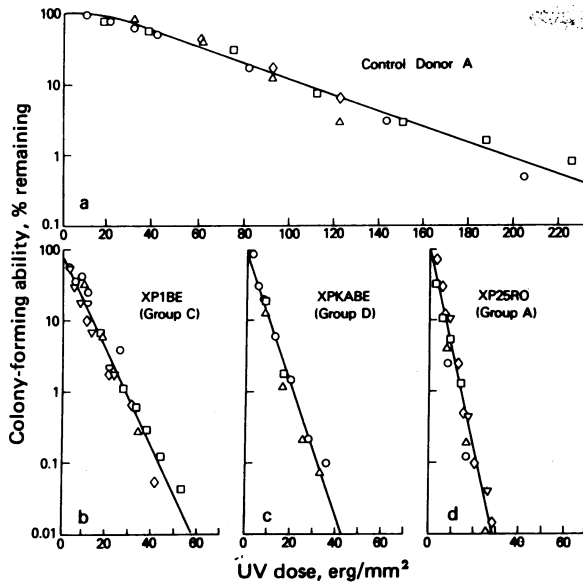


FIG. 1. Lack of effect of colony-forming efficiency of unirradiated fibroblasts on their post-UV CFA. For each strain, the points represented by a given symbol are from one experiment. The colony-forming efficiencies of the unirradiated fibroblasts are as follows: control donor A (CRL 1221): □, 9.6%; ○, 21%; ◇, 44%; △, 48%; XP1BE: □, 2.2%; ○, 3.4%; ◇, 11%; △, 14%; ▽, 18%; ⊕, 26%; XPKABE: □, 3.4%; ○, 3.5%; △, 16%; XP25RO: □, 5.3%; ○, 24%; ◇, 25%; △, 50%; ▽, 56%.

by averaging the colony-forming efficiencies for each group of dishes counted at that dose. The post-UV colony-forming ability (CFA) was calculated by dividing the colony-forming efficiency of the irradiated cells at a given dose by the colony-forming efficiency of that strain's unirradiated cells in the same experiment. A total of 96 experiments were performed, and the results for each strain were obtained from two to seven experiments, each performed on different days.

The post-UV CFA curve for each strain was drawn on semilogarithmic coordinates through all the points obtained for that strain. The presence and extent of a shoulder, i.e., a short initial portion of the curve having a slope substantially different from that of the ensuing long exponential portion of the curve, were determined by eye. Straight lines were fit by the method of least squares through all the points along the long exponential portions of the curves. A  $D_0$  value was obtained from the long exponential portion of each curve and represents the UV dose required to reduce the CFA from any point on that portion of the curve to 37% ( $1/e$ ) of that point. Statistical comparisons of these straight lines were performed using standard techniques (13). These straight lines fit the data well, having correlation coefficients that ranged from 0.94 to 0.99.

RESULTS

The colony-forming efficiency of unirradiated fibroblasts differed among the strains in an experiment and, for any given strain, differed from one experiment to another. The overall range of unirradiated colony-forming efficiencies from all of our experiments with XP strains was 0.43–56%; the overall range for control donor strains was 0.27–48%. As Fig. 1 shows, the unirradiated colony-forming efficiency had no discernible effect on the XP or control donor strain's post-UV CFA.

Fig. 2 shows the post-UV CFA for each of five control donor strains from five unrelated normal individuals aged 3–92 years. The initial portion of the post-UV CFA curve for each of these strains, extending to about 25–30  $\text{erg}/\text{mm}^2$ , has a slope sub-

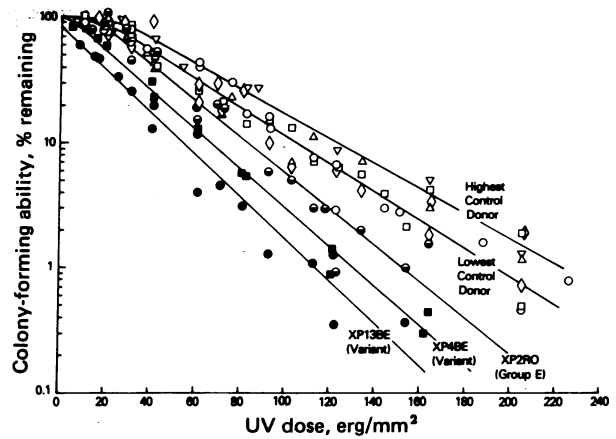


FIG. 2. Reduced post-UV CFA of XP variant and group E fibroblast strains. The exponential portion of each curve was fitted by the method of least squares through all the points along its course obtained from several ( $n$ ) experiments. Control donors: ▽, highest control donor, CRL 1121,  $n = 2$ ; ◇, CRL 1119,  $n = 4$ ; △, CRL 1116,  $n = 2$ ; □, CRL 1141,  $n = 4$ ; ○, lowest control donor, CRL 1221,  $n = 4$ ; XP group E: ⊕, XP2RO,  $n = 4$ ; XP variants: ■, XP4BE,  $n = 2$ ; ●, XP13BE,  $n = 3$ . One point above 240  $\text{erg}/\text{mm}^2$  for control donor CRL 1119 is not shown.

stantially less steep than that of the long exponential portion of the curve which follows, thus creating a "positive" shoulder. The long exponential portions of these curves have  $D_0$ s that range from 43.3 to 38.2  $\text{erg}/\text{mm}^2$  (Table 1), and the extrapolation of these portions to zero-dose (not shown in Fig. 2) intersects the ordinate between 100 and 200% (i.e., they have extrapolation numbers between 1.0 and 2.0). As determined by the Student  $t$  test for comparison of slopes, none of the slopes for the control donor curves is significantly different from any other ( $P > 0.05$  in all cases). But, as shown by tests for coincidence, the highest control donor curve is significantly different ( $P < 0.005$ ) from the lowest control donor curve, indicating that these two control donor strains have significantly different levels of post-UV CFA throughout the dose-range that was used to determine the exponential portions of their curves.

Strain XP2RO, from the Group E XP patient (3), has the highest post-UV CFA of all XP strains tested (Fig. 2). The curve for this strain has a positive shoulder indistinguishable from that of the control donor curves, but its exponential portion, having a  $D_0$  of 29.7  $\text{erg}/\text{mm}^2$  (Table 1), is significantly steeper ( $P < 0.01$ ) than that of the lowest control donor.

The lowermost two curves in Fig. 2 are for strains from two unrelated XP variants, XP4BE and XP13BE (1). The slopes of these variant curves, which have  $D_0$ s of approximately 26  $\text{erg}/\text{mm}^2$  (Table 1), are not significantly different from one another ( $P > 0.10$ ) or from the slope of the exponential portion of the group E strain's curve ( $P > 0.10$ ). However, neither of the variants' curves has a definite shoulder, and, as shown by tests for coincidence, each of the variant strains has a significantly lower level of post-UV CFA than the group E strain ( $P < 0.001$ ). The difference between the two variant curves, as determined by the test for coincidence, is not significant ( $0.05 < P < 0.10$ ).

Results for strains from the other XP groups are shown in Fig. 3. All of these strains have significantly lower post-UV CFA than any of the strains in Fig. 2. Among the six group C strains tested (Fig. 3A), we found two distinctly different types of post-UV CFA curves. Strains XP2BE and XP9BE are from siblings; their curves and that of strain XP10BE, from an unrelated patient, have small positive shoulders (up to 5–10  $\text{erg}/\text{mm}^2$ ) followed by exponential portions with  $D_0$ s of approxi-

Table 1. Identity and characterization of the fibroblast strains studied

Strains <sup>ab</sup>	$D_0$ values, <sup>c</sup> erg/mm <sup>2</sup>	Donors' neurological abnormalities <sup>d</sup>
Control donors (100%)		
CRL 1121	43.3	0
CRL 1119	44.9	0
CRL 1116	44.2	0
CRL 1141	41.3	0
CRL 1221	38.2	0
XP group E (>50%)		
XP2RO	29.7	0
XP variants (100%)		
XP4BE	27.5	0
XP13BE	25.2	0
XP group C (10–20%)		
XP2BE <sup>e</sup>	10.1	0
XP9BE <sup>e</sup>	10.4	0
XP10BE	10.3	0 <sup>f</sup>
XP4RO	8.1	0
XP3BE	8.0	0
XP1BE	6.5	0
XP group A (<2%) <sup>gh</sup>		
XP8LO <sup>h</sup>	17.4	0
XP1LO <sup>i</sup>	8.8	0
XP12BE	7.0	+
XP6TO	3.1	++++
XP4LO	3.0	++++
XP25RO <sup>j</sup>	3.1	++++
XP26RO <sup>j</sup>	2.8	++++
XPKFSF	2.9	++++
XP group D (25–50%)		
XP5BE <sup>k</sup>	4.7	+++
XP6BE <sup>k</sup>	4.2	+++
XPKABE	4.7	+++
XP7BE <sup>l</sup>	4.3	+++
XP2NE <sup>m</sup>	4.4	+++
XP3NE <sup>m</sup>	3.9	+++ <sup>n</sup>
XP17BE	3.8	+++

<sup>a</sup> Classification of the XP variants was by determination of unscheduled DNA synthesis (1). Classification of the excision-deficient XP strains into complementation groups A, C, D, and E was by cell fusion studies.

<sup>b</sup> The UV-induced unscheduled DNA synthesis of each strain, expressed as a percent of the normal rate, was determined in our laboratory as described elsewhere (1, 11).

<sup>c</sup> Each  $D_0$  value was obtained from the long exponential portion of the appropriate curve in Figs. 2 or 3 and represents the UV dose required to reduce the CFA from any point on that portion of the curve to 37% of that point.

<sup>d</sup> 0, no known neurological abnormalities; +, +++, +++++, as in Fig. 4. The detailed neurological status of most of the XP patients has been described (1). The neurological status of the other patients was obtained from the following references: XP2RO and XP4RO (8); XP8LO (7, 8); XP1LO (14); XP6TO (9); XP4LO (14); XP25RO and XP26RO (8); XPKFSF (15); XP2NE and XP3NE (6, 7).

<sup>e</sup> Patients XP2BE and XP9BE are siblings (1).

<sup>f</sup> XP10BE is the only patient in group C with any known neurologic abnormality (1). Because this abnormality was only a dull-normal IQ, we have previously (1, 4) considered her abnormality to be independent of her XP.

<sup>g</sup> Strains XP1LO, XP12BE, XP25RO, and XPKFSF have been shown (16) to have 0.4–1.3% of the normal rate of unscheduled DNA synthesis.

<sup>h</sup> Strain XP8LO is the only group A strain found to have more than 2% of the normal unscheduled DNA synthesis rate (7, 17). Its rate is 30–35% of normal.

<sup>i</sup> This strain was found to be contaminated with *Mycoplasma hyorhinis*.

mately 10 erg/mm<sup>2</sup> (Table 1). These three curves do not differ significantly from one another either in slope or in level of post-UV CFA ( $P > 0.05$  in all cases). The curves for strains from three other unrelated group C patients (XP3BE, XP4RO, and XP1BE) show no evidence of a positive shoulder, having extrapolation numbers between 0.50 and 0.80. The slopes of these three curves, whose  $D_0$ s range from 6.5 to about 8.0 erg/mm<sup>2</sup> (Table 1), are significantly steeper ( $P < 0.02$ ) than those of the previously described group C strains, and one of the three, that for XP1BE, is significantly steeper ( $P < 0.01$ ) than the other two.

The results for six group D strains, representing four kindreds, are shown in Fig. 3B. An additional group D strain, XP7BE (not shown in Fig. 3B), has been studied, bringing the total kindreds studied to five. All these group D patients have numerous neurological abnormalities, which usually become clinically well-manifested between 7 and 12 years of age, in contrast to the absence of neurological abnormalities in the group C, XP variant, and group E patients, most of whom are already adults. The group D curves have no discernible shoulders, and have an average  $D_0$  of approximately 4.3 erg/mm<sup>2</sup> (Table 1). Each curve is significantly steeper ( $P < 0.001$ ) than the steepest group C curve in Fig. 3A. From the data obtained, the slopes of the group D curves could not be shown to differ significantly from one another (i.e.,  $P > 0.05$ ) except in the case of the curve for XP17BE, which is significantly steeper than that for XPKABE, and that for XP5BE ( $P < 0.05$  in both instances). However, as shown by tests for coincidence between the curves, each of strains XP17BE, XP3NE, and XP2NE has significantly lower post-UV CFA ( $P < 0.02$ ) than strains XPKABE, XP6BE, XP5BE, or XP7BE. There are no significant differences in post-UV CFA among the latter four strains, two of which (XP5BE and XP6BE) are from siblings. Among the other three strains, XP17BE differs significantly ( $P < 0.01$ ) from XP2NE, but the siblings, XP2NE and XP3NE, do not differ significantly ( $P > 0.10$ ) from one another.

Results for eight group A strains, representing seven kindreds, are shown in Fig. 3C. Neither patient XP8LO (currently 6 years old) (7) nor patient XP1LO (39 years old) (14) has any neurological abnormalities. Patient XP12BE (currently 12 years old) has two such abnormalities, areflexia and an abnormal electroencephalogram (1). Patients in the remaining four group A kindreds had numerous neurological abnormalities by 7 years of age. The group A strain with the highest post-UV CFA, XP8LO, has a  $D_0$  of 17.4 erg/mm<sup>2</sup>, significantly higher than that of all other group A strains. The data for this strain do not clearly indicate whether or not its curve has a small positive shoulder (5 erg/mm<sup>2</sup> or less). Two strains with intermediate post-UV CFA, XP1LO and XP12BE, give curves that have  $D_0$ s of 8.8 and 7.0 erg/mm<sup>2</sup>, respectively, for the long exponential portions of their curves. These slopes are significantly different from each other and from the slopes of all other group A strains. The slopes of XP12BE and of XP1BE (the lowest group C curve) are not significantly different ( $0.20 < P < 0.30$ ), but, as

<sup>j</sup> Patients XP25RO and XP26RO are siblings (8).

<sup>k</sup> Patients XP5BE and XP6BE are siblings (1).

<sup>l</sup> This strain was found to be contaminated with *Mycoplasma arginini*.

<sup>m</sup> Patients XP2NE and XP3NE are siblings (6).

<sup>n</sup> Mild progressive deafness, slurring of speech, and trembling of the limbs were noted by 22 years of age, and absence of tendon reflexes was noted at 23 years of age; therefore, the age of onset of these neurological abnormalities is unknown. The patient's brother, XP2NE, had slurring of speech by 10 years of age (6).

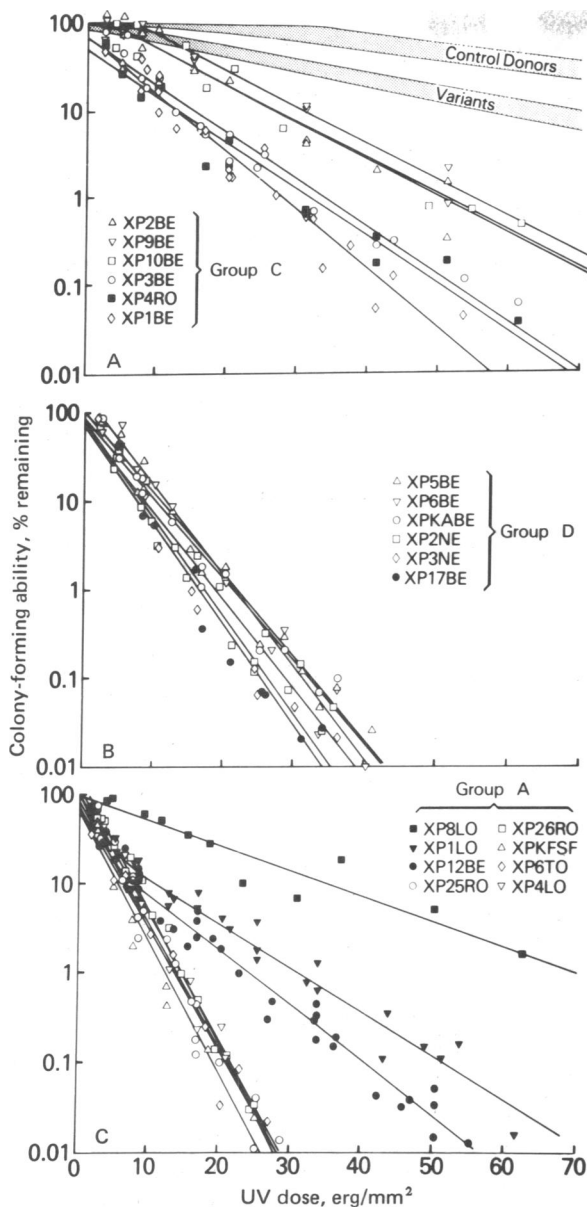


FIG. 3. Post-UV CFA of XP fibroblast strains from complementation groups C (A), D (B), and A (C). The shaded areas at the top of A encompass the lines in Fig. 2 representing the post-UV CFAs of the control donors and of the XP variants. The points were obtained from two to seven experiments performed with each strain. One point with a CFA below 0.01% is not shown for each of the following: XP6BE and XP17BE (B); XP12BE, XP25RO, and XP26RO (C). Siblings: XP2BE, XP9BE; XP5BE, XP6BE; XP2NE, XP3NE; XP25RO, XP26RO.

shown by the test for coincidence, the long exponential portion of the XP12BE curve is significantly lower ( $P < 0.001$ ) than that for XP1BE. The extrapolation numbers (not shown) for the long exponential portions of the XP1LO and XP12BE curves are 0.33 and 0.30, respectively. Each curve has an initial portion (from 0 to about 8 erg/mm<sup>2</sup>) that is steeper than the long exponential portion and thus creates a "negative" shoulder. Within these initial portions the curves for XP1LO and XP12BE are indistinguishable from the curves for the remaining five group A strains shown in Fig. 3C. These five strains have post-UV CFA curves with no discernible shoulders. With one exception, their  $D_{0.5}$ s of approximately 3.0 erg/mm<sup>2</sup> (Table 1) are all significantly lower ( $P < 0.05$ ) than those of any group D strain tested.

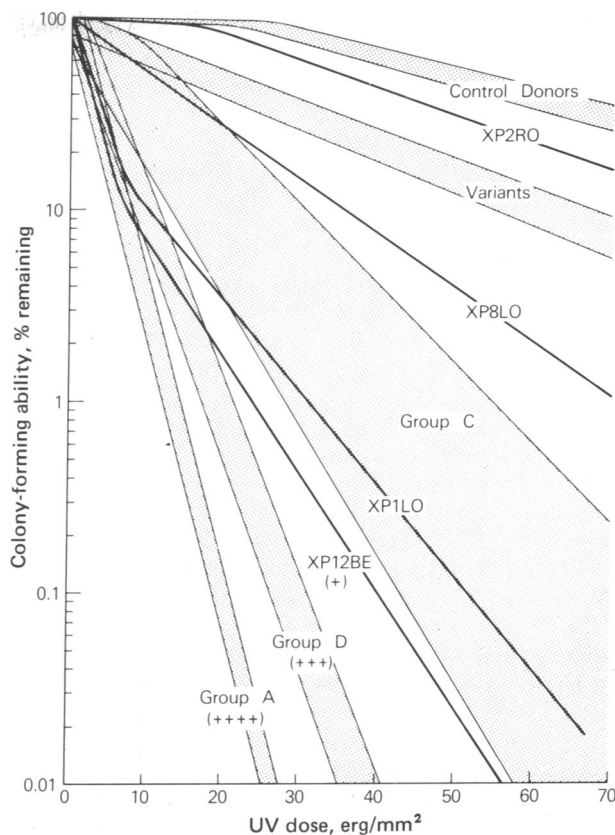


FIG. 4. Heterogeneity of post-UV CFA among group A strains and the relationship between neurological abnormalities of XP patients and the post-UV CFA of their fibroblasts. The shaded areas for the control donors' and XP variants' strains are as in Fig. 3A; the shaded area for the strains of group C encompasses the curves in Fig. 3A; the shaded area for the strains of group D encompasses the curves in Fig. 3B and the curve for strain XP7BE; the shaded area for the group A strains encompasses the lowermost five curves (open symbols) in Fig. 3C. The number and age of onset of neurological abnormalities are indicated by the following symbols: +++++, numerous abnormalities clinically manifested by 7 years of age; +++, numerous abnormalities clinically manifested between 7 and 12 years of age; +, areflexia and abnormal electroencephalogram present by 10 years of age.

The one exception, strain XP6TO, has a slope that, compared to that for strains XP17BE and XP3NE (the group D strains with the steepest curves), is not significantly different ( $0.05 < P < 0.10$  in both cases); however, tests for coincidence show that strain XP6TO does have significantly lower post-UV CFA than either of these group D strains ( $P < 0.01$ ). The slopes of the five lowermost group A curves do not differ significantly from one another ( $P > 0.05$  in all cases), but tests for coincidence show that one curve, that for XPKFSF, differs significantly in post-UV CFA from the curves for the siblings, XP25RO and XP26RO ( $P < 0.05$ ). No other significant differences among these five curves are shown.

In Fig. 4, the marked heterogeneity of post-UV CFA among the group A strains can be seen in relation to the post-UV CFA of the other XP groups. Fig. 4 also illustrates the relationship between the post-UV CFA of an XP patient's fibroblasts and that patient's clinical neurological status described in the literature (references in footnote <sup>d</sup> of Table 1). In all cases studied, the fibroblasts of any patient with known XP-associated neurological abnormalities were found to have lower post-UV CFA and, therefore, a greater UV sensitivity than those of any patient with no such abnormalities.

## DISCUSSION

The difference in ability of XP and control donor fibroblasts to form colonies after UV irradiation reflects the different capacities of the cells to repair their damaged DNA to the biologically functional level required to complete repeated cycles of cell division. The correlation we have found between the XP fibroblasts' post-UV CFA and the patient's neurological abnormalities leads us to the following hypothesis. The damage induced in the fibroblast DNA by the UV radiation requires the same processes for its repair as the currently unknown damage we postulate must occur in the DNA of the central nervous system of both XP and normal individuals during embryonic and postnatal life. All dividing normal cells studied (1), including neurons (18), can perform excision repair at some stage of their development. Therefore, even if both normal and XP postmitotic neurons lack excision repair, dividing neurons in the normal fetus can presumably repair their DNA, thus eliminating the type of damage that would accumulate in neurons of an XP fetus before birth. We postulate further that the degrees of biologically effective repair of damaged DNA in the nervous system of the XP patients mirror the relative post-UV CFAs of the patients' fibroblasts in our studies *in vitro*. Thus, the patients whose fibroblasts have the least post-UV CFA also have the least functional repair of the damaged DNA in their nervous systems. As further insults to the DNA of the XP neurons (or neuron-supporting glial cells) occur, the cells' DNA can no longer provide sufficient, accurate informational instruction for the neurons' vital transcription of RNA. As the neurons lose the capacity to synthesize critical enzymes or other molecules necessary for their survival, they die prematurely, and the neurological abnormalities of XP (1) result. Furthermore, we postulate that in normal individuals the gene products from the DNA repair cistrons A and D must have at least a certain level of DNA repair capacity in order to maintain the functional integrity of the nervous system. Our studies, however, do not indicate whether or not DNA excision repair processes play an etiologic role in "normal" aging of the human nervous system.

For 13 patients whose cells were used in the present study, we have obtained reliable clinical histories (1) regarding the presence or absence of acute sun sensitivity, i.e., skin blistering and/or severe erythema after a sun exposure that would not produce such results in normal individuals. Patients whose fibroblasts had markedly decreased post-UV CFA had histories of acute sun sensitivity. Thus, all five Bethesda patients in group D had acute sun sensitivity and  $D_0$  values less than 5 erg/mm<sup>2</sup>

(Table 1). No acute sun sensitivity was present in either of the two variants or in the three group C patients (XP2BE, XP9BE, XP10BE) all of whom had  $D_0$  values above 10 erg/mm<sup>2</sup> (Table 1). Patients whose fibroblasts had  $D_0$  values from 6.5–8 erg/mm<sup>2</sup> either had (XP12BE, XP3BE) or did not have (XP1BE) acute sun sensitivity. The results of our post-UV CFA studies, therefore, appear to correlate not only with the XP patients' neurological status but also with at least one of their cutaneous abnormalities.

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