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Cells from an immunodeficient patient (46BR) with a defect in DNA ligation are hypomutable but hypersensitive to the induction of sister chromatid exchanges

(human fibroblasts/DNA repair/mutation/Okazaki fragments)

LEIGH M. HENDERSON*, COLIN F. ARLETT†, SUSAN A. HARCOURT†, ALAN R. LEHMANN†, AND BERNARD C. BROUGHTON†

*Centre for Medical Research, School of Biological Sciences, and †Medical Research Council Cell Mutation Unit, University of Sussex, Falmer, Brighton, Sussex BN1 9RR, United Kingdom

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ABSTRACT **A fibroblast cell strain, 46BR, derived from an immunodeficient patient is hypersensitive to the lethal effects of a wide range of DNA-damaging agents. It is also defective in strand-break rejoining after treatment with dimethyl sulfate and UV light. The present study shows that the cells have a defect in joining Okazaki-type fragments during DNA replication, supporting the interpretation that the basic defect is in ligation of DNA strands. The baseline level of sister chromatid exchange is slightly higher than in normal cells but it does not approach that of Bloom's syndrome or dyskeratosis congenita cells. Sensitivity to the induction of sister chromatid exchange and the hypersensitivity to the lethal effects of a set of DNA-damaging agents are correlated, implying that the basic defect influences both end points in a similar manner. No 6-thioguanine-resistant mutants could be induced by either γ- or UV-irradiation in these cells, suggesting that error-prone repair pathways for damage induced by these agents may contain a common ligation step in human cells.**

Previous studies with the fibroblast cell strain 46BR, derived from an immunodeficient patient (1), have shown hypersensitivity to the lethal effects of a wide range of DNA-damaging agents (2). In particular the cells were hypersensitive to γ- and UV-irradiations, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), methyl methanesulfonate, *N*-methyl-*N*-nitrosourea (MNU), dimethyl sulfate (Me_2SO_4), and ethyl methanesulfonate but not *N*-ethyl-*N*-nitrosourea (ENU). These results suggested that the cells might be defective in a common, late step of excision repair. Biochemical studies showed that, following treatment with Me_2SO_4 (3) or UV (4), more strand breaks persisted in 46BR cells than in normal cells. In addition, the amount of repair synthesis after Me_2SO_4 treatment was greater in 46BR cells than in normal cells (3). 46BR cells are also hypersensitive to the lethal effects of 3-aminobenzamide, which is an inhibitor of ADP-ribosyl transferase, an enzyme that is able to modify the activity of DNA ligases (5). Taken together, these observations led to the suggestion that 46BR cells might be defective in DNA ligase activity (6). Further support for the hypothesis of a ligation defect is provided in the present work in which we show that joining of Okazaki-type fragments during DNA replication is delayed in 46BR cells.

Xeroderma pigmentosum (XP) cells defective in excision repair are hypersensitive to the induction of sister chromatid exchanges (SCEs) by UV light although the spontaneous level is normal (7). Ataxia-telangiectasia (A-T) cells have normal levels of baseline and mitomycin C-, ethyl methanesulfonate-, x-ray-, or adriamycin-induced SCEs (8, 9). These results and additional studies on Fanconi's anemia (10–12) and Bloom's syndrome (13) have failed to show a clear association of any known DNA repair process with SCE formation. We examined the induction of SCEs in 46BR cells, and we show that the induction of SCEs by several different agents is elevated.

We also examined the response of 46BR to the mutagenic effects of UV- and γ-irradiation. We have not been able to detect any induced mutants.

MATERIALS AND METHODS

Cell Strains. Ten cell strains have been used in this study. Their characteristics and the phenotype of donor individuals are summarized in Table 1.

Culture Conditions. All cell strains were cultured in 5% CO_2/95% air incubators in Eagle's minimal essential medium supplemented with 15% fetal calf serum (14).

Treatments. γ-Irradiation was provided by a cobalt-60 source and 254-nm UV light from an Hanovia germicidal lamp. UV doses were monitored with a Latarjet meter. MNNG and MNU were made up in McIlvaine's buffer at pH 5, and cultures were treated for 1 hr and 15 min, respectively, in complete medium. ENU was made up in absolute alcohol, and cells were treated for 15 min in complete medium. Cells were treated with Me_2SO_4 for 20 min in complete medium.

DNA Replication Intermediates. Cells that had been plated at 1.5×10^5 per 5-cm dish and incubated for 3 days were pulse-labeled with 40 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine per ml for 5, 10, or 15 min. In some experiments a 10-min pulse-label was followed by further incubation in the absence of radioactivity. The size of the pulse-labeled DNA was measured by centrifugation in alkaline sucrose gradients. The cells were first γ-irradiated to facilitate release of the nascent DNA strands on subsequent lysis of the cells on top of the 5–20% alkaline sucrose gradients (15). The gradients were centrifuged at 38,000 rpm for 70 min, fractions were collected, and the acid-insoluble radioactivity was measured as described (15, 16).

Sister Chromatid Exchanges. Cells were set up at 1.2×10^5 cells per 25-cm^2 flask. After 48 hr a medium change was performed, and at that time freshly prepared chemicals were added. Following treatment for appropriate periods, the medium was removed and replaced with fresh medium containing bromodeoxyuridine (10 μM) and deoxycytidine (10 μM). After incubation in the dark for 48 hr, colchicine

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Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MNU, *N*-methyl-*N*-nitrosourea; Me_2SO_4, dimethyl sulfate; ENU, *N*-ethyl-*N*-nitrosourea; XP, xeroderma pigmentosum; A-T, ataxia-telangiectasia; SCE, sister chromatid exchange.

Table 1. Cell strains and characteristics of donors

Cell strain	Phenotype of donor	Comments
1BR*	Normal	
48BR†	Normal	
54BR†	Normal	
2BI‡	Normal	
GM730§	Normal	
HG1376¶	Normal	
AT4BI‡	A-T	γ-ray hypersensitive
XP2BI‡	XP (complementation group G)	UV hypersensitive
XP4LO‖	XP (complementation group A)	UV hypersensitive
46BR**	Immunodeficient	Hypersensitive to lethal effects of many DNA-damaging agents

Source of cell strains:
*C. F. Arlett, Brighton, U.K.
†C. Tredgold, Brighton, U.K.
‡A. M. R. Taylor, Birmingham, U.K.
§A. Greene, Camden, NJ.
¶J. German, New York.
‖P. Hall-Smith, Brighton, U.K.
**D. Webster, Harrow, U.K.

(final concentration, 1 μM) was added for a further 6 hr before harvesting by standard cytological procedures. Differentiation of sister chromatids was achieved by the method of Goto *et al.* (17). In each experiment at least 20 metaphases, each containing at least 40 chromosomes, were analyzed for SCEs for each data point. In most experiments the 46BR cell strain was treated in parallel with a normal cell strain, and the experiments on 46BR were repeated 2 or 3 times for each DNA-damaging agent.

Mutation. The technique for detecting mutation in human fibroblast cultures followed that described by Cox and Masson (18), which utilizes the Sterilin-designed bulk culture vessels. We used 6-thioguanine (Sigma) to select for resistant mutants. With 46BR as with any new cell strain, the toxic response to a range of concentrations of 6-thioguanine was assessed; 2.5 μg/ml was found to be a suitable concentration for selection of resistant mutants. While cells are maintained routinely in medium containing 15% fetal calf serum, for survival platings and for selection of 6-thioguanine-resistant mutants in bulk culture vessels, newborn calf serum (GIBCO-Biocult) was used in the interests of economy (14). The design of mutation experiments with UV- or γ-rays has been detailed elsewhere (19).

RESULTS

DNA Replication Intermediates. DNA ligases are involved in DNA replication, one of their functions being the ligation of Okazaki-type replication intermediates. These intermediates, about 200 nucleotides in length in mammalian cells (20), are extremely transient in cells incubated at 37°C, and they can be detected only with very short pulse-labels (less than 1 min) (e.g., see ref. 21). With longer labeling times, they are not detected. This is confirmed in our experiments with normal human fibroblasts (Fig. 1 *A* and *B*). With 5- or 10-min pulse-labels, the labeled DNA formed a broad band across the center of the alkaline sucrose gradients. Such a distribution is characteristic of replicating DNA in asynchronous cells and represents growing DNA strands whose sizes range from 10 to 100 × 10^6 daltons. In contrast most of the DNA from 46BR cells labeled under identical conditions was found at the top of the sucrose gradients (Fig. 1 *C* and *D*), representing DNA of much lower molecular mass. Thus,

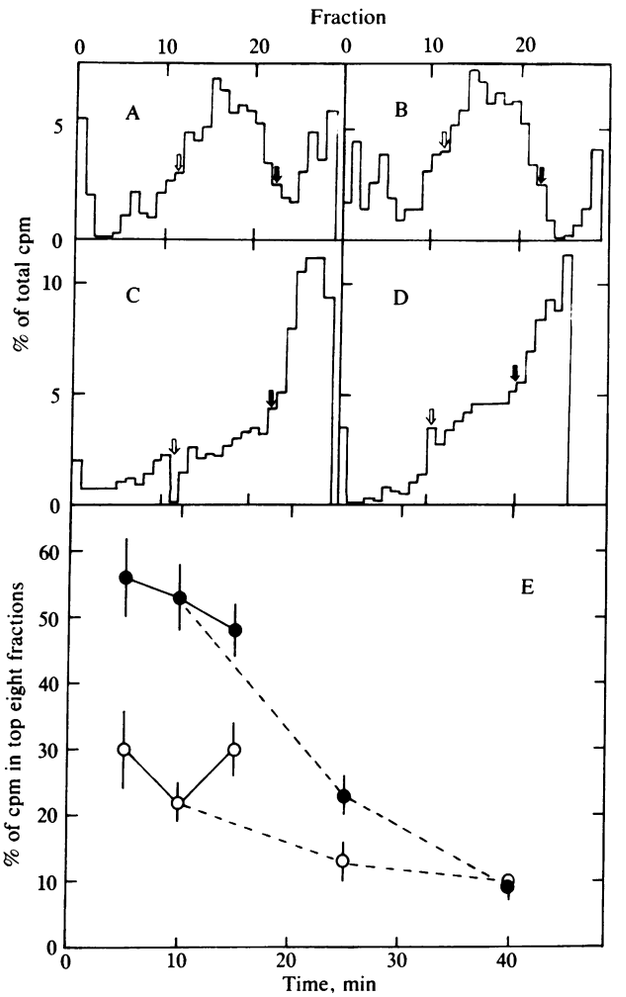

FIG. 1. Replication intermediates. Normal 1BR (*A* and *B*) or 46BR (*C* and *D*) cells were pulse-labeled for 5 min (*A* and *C*) or 10 min (*B* and *D*) with [^{3}H]thymidine. The relative size of the labeled DNA strands was measured on alkaline sucrose gradients, sedimentation being from right to left. (*E*) Pooled data from a variety of normal cells (○) or 46BR cells (●). Cells were pulse-labeled for various times (—) or were labeled for 10 min followed by chases as indicated (——). Results show means ± SEM of two-to-seven experiments. Arrows indicate the positions of DNA molecules whose molecular masses are 10 × 10^6 (open symbols) and 100 × 10^6 (closed symbols) daltons.

46BR cells showed a clear delay in the joining of replication intermediates. That this delay was only transient is shown in Fig. 1*E*. Cells labeled with [^{3}H]thymidine for 10 min were chased for various periods of time, and the DNA was centrifuged on alkaline sucrose gradients. The fraction of the total radioactivity in the top eight fractions (molecular mass < 8 × 10^6 daltons) is plotted as a function of chase time. Immediately after the pulse-label, much more labeled DNA was at the top of the gradient in 46BR than in normal cells; after a 30-min chase, this DNA had attained a molecular mass similar to that in normal cells.

Sister Chromatid Exchanges. A higher baseline level of SCEs in 46BR, average 11.3 ± 0.6 SCEs per metaphase (12 experiments) compared with average control values between 7.1 ± 0.8 (1BR; five experiments) and 8.4 ± 0.5 (GM730; three experiments) was noted and may indicate an increased sensitivity to bromodeoxyuridine. The induction of SCEs in 46BR and a set of normal cell strains by five DNA-damaging agents are shown in Fig. 2. Strain 46BR was more sensitive than normal cells to SCE induction by UV light. The

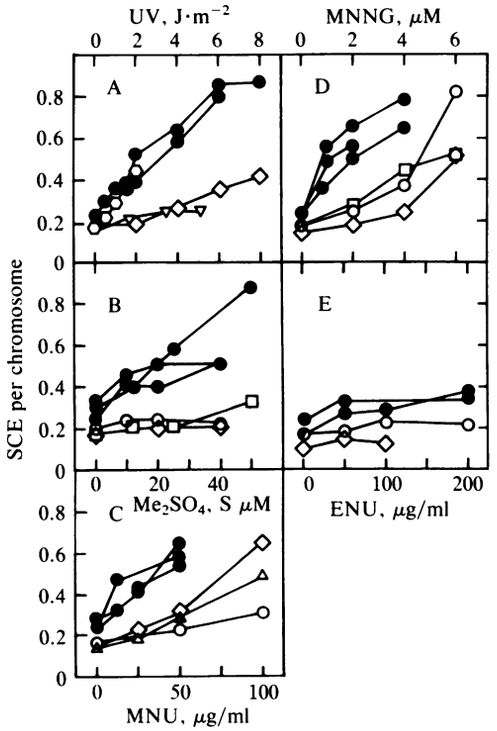

FIG. 2. Induction of SCEs by UV (two experiments) (*A*), Me_2SO_4 (three experiments) (*B*), MNU (three experiments) (*C*), MNNG (three experiments) (*D*), and ENU (two experiments) (*E*). ◇, 1BR cell line; ▽, HG1376; ○, XP2BI; ●, 46BR; ○, GM730; □, 54BR; △, 48BR.

hypersensitivity was similar to that of XP cell strain XP2BI, which was included to scale the response of 46BR. In the case of Me_2SO_4, no SCEs were induced in two experiments with the normal cell strains 1BR and GM730 at doses that did induce SCEs in strain 46BR. In a third experiment, SCEs were induced in the normal cell strain 54BR, but this induction was much less than in strain 46BR, the calculated regression coefficients being 0.13 for 54BR and 0.56 for 46BR. With MNU the responses of three normal cell strains were not significantly different from each other. A regression coefficient was determined from the pooled 46BR results (three experiments) and compared with the pooled values for the normal cells. The calculated regression coefficient for 46BR (0.39) was considerably higher ($P = 0.001$) than that for the normal cell strains (0.15). Strain 46BR was also clearly hypersensitive to the induction of SCEs by MNNG. For ENU, a small but significant increase in SCEs was observed in both strain 46BR and one normal cell strain, GM730.

Mutation. 46BR cells appeared to be slightly more sensitive to 6-thioguanine than do normal cells (Fig. 3). We examined the induction of mutations to 6-thioguanine resistance by UV in three separate experiments (Fig. 4*A*). In only one of six dose points was there any indication of induction of mutants. The data for normal cell strain GM730 were obtained in the same experiment as the 46BR data. The response of GM730 falls in the normal range of UV mutability obtained in a large number of earlier experiments (19) (shown in Fig. 4*A* by the shaded region). Data from a separate earlier experiment with XP strain XP4LO is included to scale the response. Actual survival data and numbers of mutant colonies obtained for one experiment with UV-irradiation (corresponding to triangles in Fig. 4*A*) and one experiment with γ-irradiation (corresponding to squares in Fig. 4*B*) are shown in Table 2.

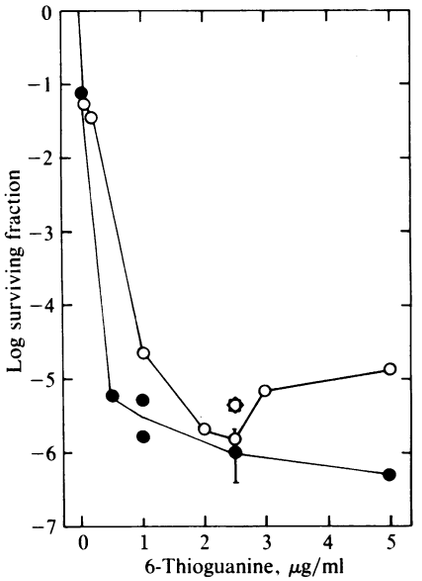

FIG. 3. Resistance to 6-thioguanine of strain 46BR (●) and normal strain 2BI (○). The mean value for a large number of human cell strains (▱) is also shown (from ref. 19). Repeated observations were made at 2.5 μg of 6-thioguanine per ml, allowing estimates of the standard error.

With γ-irradiation (Fig. 4*B*), three experiments were performed with 46BR cells. In the first and second experiments there was no induction of mutants, and no mutants at all were recovered in a third experiment when 8.9×10^6 viable cells were tested. In the second and third experiments, γ-irradiation did induce mutants in normal cell strains 54BR and GM730. Data are provided for a third normal cell strain, 48BR, which was tested in an independent experiment but during the same time-period in which the other experiments were performed. The data on mutation induction in XP4LO after γ-irradiation are also included. Although the spontaneous mutation frequencies for these four cell strains (54BR,

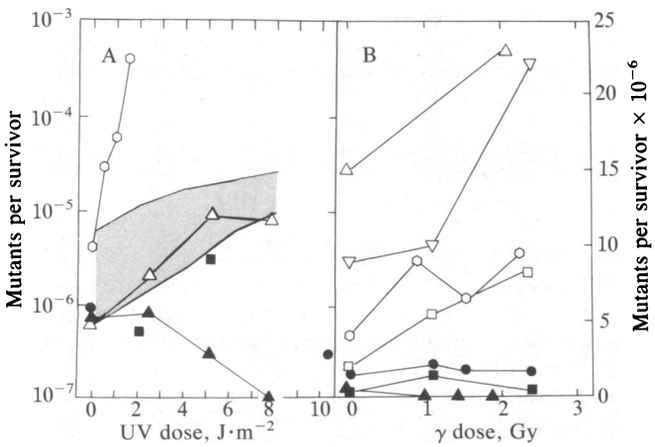

FIG. 4. Induction of mutation to resistance to 6-thioguanine at 2.5 μg/ml by 254-nm UV-irradiation (*A*) and γ-irradiation (*B*). (*A*) Three separate experiments are shown for strain 46BR (●, ■, and ▲) and one each for normal cell strain GM730 (△) and strain XP4LO (○). The data are plotted on a logarithmic scale. The shaded section indicates the normal range of mutability. (*B*) Three separate experiments with strain 46BR are shown for γ-irradiation (●, ■, and ▲) and one each for three normal cell strains GM730 (△), 48BR (▽), and 54BR (□), and one for strain XP4LO (▱). Identical open or closed symbols indicate paired observations. The data are plotted on a linear scale.

Table 2. Induction of 6-thioguanine-resistant mutants by UV- and γ-irradiation

Cell strain	Treatment	Initial survival, %	Viable cells tested × 10^6	No. of mutants	Mutation frequency per survivor × 10^{6*}
46BR	0 Gy	100	11.0	3	0.3
	1.1 Gy	31.1	6.4	10	1.5
	2.2 Gy	12.2	5.9	1	0.1
54BR	0 Gy	100	4.2	12	2.8
	1.1 Gy	60†	5.2	38	5.3
	2.2 Gy	31†	6.4	56	8.6
46BR	0 J·m^{-2}	100	10.8	8	0.7
	2.6 J·m^{-2}	27.8	5.6	5	0.8
	5.3 J·m^{-2}	11.3	6.3	2	0.3
	11.3 J·m^{-2}	5.6	5.4	1	0.1
54BR	0 J·m^{-2}	100	11.2	6	0.5
	2.6 J·m^{-2}	90.8	6.5	13	2.0
	5.3 J·m^{-2}	48.7	5.3	48	9.0
	7.9 J·m^{-2}	31.0	5.6	46	8.2

*Cells were tested after a 13- and 20-day expression time. In all cases the mutation frequency was obtained from populations where recovery from the lethal effects of irradiation was complete. The higher value of the two sampling times was used for the estimation of mutation frequency.

†Survival values obtained from a separate experiment.

GM730, 48BR, and XP4LO) were quite different, the induction of mutations by γ-irradiation, as shown by the slopes of the lines, was quite similar.

DISCUSSION

The observations on hypersensitivity of 46BR cells to the lethal action of an array of DNA damaging agents with differing modes of action led to the suggestion that the cells might be defective in a late step of excision repair (2). Studies of strand-break repair and sensitivity to 3-aminobenzamide implicated a defect in DNA ligase activity (6). The delay in joining of Okazaki-type fragments in 46BR cells in comparison with normal cells (Fig. 1) is also in agreement with the presence of a functional defect in DNA ligase activity. Whereas these results are entirely consistent with a defect in ligation, no direct defect in DNA ligase activity in crude extracts of 46BR cells has been detected as yet (unpublished observations). [It seems appropriate here to note an analogy with XP. Evidence obtained from whole cells strongly suggests that a UV endonuclease is defective in XP, but the activity of UV endonuclease in XP cell extracts is normal (22). The extract may not be functional if the substrate is UV-irradiated chromatin rather than DNA (22).]

The consequences of the defect in ligation in 46BR have been examined for two genetic end points: the induction of SCEs and mutation to 6-thioguanine resistance. Elevated baseline levels of SCEs have been recorded in Bloom's syndrome (13) and dyskeratosis congenita (23). Bloom's syndrome cells have a 10-fold increase in their baseline levels and may be discriminated clearly from 46BR. The 2-fold factor for dyskeratosis congenita is higher than that for 46BR, where a 50% increase was found, and the differential diagnosis of the patient does not fit this condition (1).

The sensitivity of 46BR cells to the induction of SCEs by an array of DNA-damaging agents and the hypersensitivity to the lethal effects of these same agents are correlated (2). The relationship between these two end points is illustrated

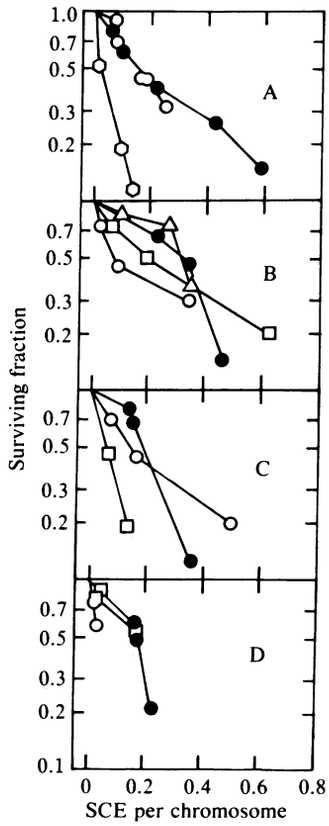

FIG. 5. Relationship between SCEs and cytotoxicity for various cell strains after treatment with 254-nm UV (*A*), MNNG (*B*), MNU (*C*), and Me_2SO_4 (*D*). ●, 46BR cell line; ○, 1BR; □, GM730; △, 54BR; ◌, XP2BI. The survival values are those published in ref. 2.

in Fig. 5. Despite the variation between the normal cell strains, the incidence of SCE at equitoxic doses is similar for strain 46BR and the normal cell strains. Since it appears unlikely that there is a causative relationship between SCE and cell death (24), these results suggest that the basic defect in strain 46BR affects both SCE formation and the induction of cell death in a similar manner. This positive correlation is to be contrasted with observations on other cell strains. De Weerd-Kastelein *et al.* (7) found that the levels of SCE induction in different XP cell strains were not correlated with their sensitivities to the lethal effects of UV. Similarly, Marshall *et al.* (25) reported that fibroblasts from Cockayne's syndrome fell into two groups with respect to the lethal effects of UV but could not be distinguished on the basis of the induction of SCE. In Fanconi's anemia a normal frequency of SCE induction follows treatment with mitomycin C despite hypersensitivity to its lethal effects (26).

It is believed that SCEs occur as a consequence of the presence of unrepaired damage persisting in DNA until S phase. Models have been proposed involving exchanges at the replication fork (27) or at the junctions of adjacent replicon clusters (28). Strain 46BR shows a persistence of alkylation and UV-induced strand breaks, which is presumed to be due to a defect in ligation. The presence of strand breaks could either increase the chance of SCEs occurring at the sites of damage or delay the replication fork progression and, consequently, increase the frequency of exchange at the junctions of replicon clusters as proposed by Painter (28).

Strain 46BR has some features in common with a Chinese hamster ovary cell line, EM9, with a broad range of hypersensitivities to mutagens, an increased sensitivity to

the induction of SCEs, and a defect in DNA strand-break rejoining (29). However, the EM9 cells are different from 46BR cells in showing a high baseline level of SCEs.

Arlett and Harcourt have claimed elsewhere (30) that A-T fibroblast cell strains are hypomutable to 6-thioguanine resistance by γ-irradiation but normal in their response to mutation induction by UV. XP cells are hypermutable in response to UV but normal with respect to γ-irradiation (30). Cells derived from patients with familial melanoma have recently been shown to be hypomutable when treated with UV (31). 46BR cells are hypomutable with both agents and thus appear to be unique. They may thus be defective in a step of error-prone repair (32) common to both UV and γ-irradiation. An involvement of DNA ligase in error-prone repair has been implied previously from studies with bacteriophage T4, in which UV- and nitrous acid-induced mutation could be eliminated by the presence of a temperature-sensitive ligase allele (33). The defect in ligation, demonstrated by the slow rejoining of replication intermediates in 46BR, could be in some way responsible for its hypomutability. However, it would be premature at present to speculate on a specific mechanism. The many repair events in both 46BR and normal cells must involve ligation steps; thus, it becomes necessary to postulate that error-prone repair requires either more ligation-dependent steps than other forms of repair or a specific ligase that may be defective or absent in 46BR.

Other explanations of the present mutation results must be considered. The slightly greater sensitivity of 46BR cells to 6-thioguanine may indicate perturbations of purine biosynthesis. Spontaneous mutants can, however, be recovered, albeit at a low frequency (19).

The cellular pathology of this immunodeficient patient has provided us with an example of a human cell strain defective at a late step in excision repair. It may also be an example of a cell strain with a defect in error-prone repair.

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