A HUMAN SUBJECT WITH A NEW DEFECT IN REPAIR OF ULTRAVIOLET DAMAGE

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The subject under study (11961) is a child with extreme sun sensitivity. Fibroblasts derived from the child's skin, like those from patients with the disorder xeroderma pigmentosum were hypersensitive to the lethal effects of 254 nm and 310 nm UV-irradiation. Unlike xeroderma pigmentosum cells, however, fibroblasts from our subject were not hypersensitive to the chemical mutagen N-hydroxyacetamidinofochrome salt but they were hypersensitive to ethylmethanesulfonate. Furthermore, despite the ultraviolet light sensitivity, no defects could be detected either in excision or postreplication repair of damaged DNA after UV-irradiation of 11961 cells. This again contrasted with xeroderma pigmentosum cells, which are defective in one or the other of these repair processes. On the basis of these characteristics and the clinical symptoms, we are not present able to classify this patient as having any of the known sun-sensitive syndromes.

The autosomal recessive disorder xeroderma pigmentosum (XP) is a human skin disease characterized by an extremely high incidence of sunlight-induced skin cancers [1,2]. To date, all XP cells studied have shown to exhibit defects in either excision [3] or postreplication [4] repair of DNA after UV-irradiation. The former, but not the latter defect is associated with greatly increased cellular sensitivity to the lethal effects of UV-irradiation [5–8], whereas either type of defect gives rise to an increase in the cells' mutability by ultraviolet light [9,10]. The defects in repair can be detected at the molecular and cellular levels before any permanent skin damage is observed and make it possible to detect XP individuals by annocentesis [11] or after birth, so that suitable protective procedures may be employed [1].

We wish to present here details of a sun-sensitive individual (patient No. 11961). Although his cellular response to UV is similar to that of patients with XP, several other characteristics at the cellular and molecular levels are distinct from that condition.

CLINICAL DESCRIPTION

The British male who is the subject of this study came to our notice at the age of 6 mo because of acute sunburn. He is an only child and there is no pertinent family history, except that his father is alleged to sunburn easily. The photosensitivity had been observed by the child's mother since 1 mo of age, even on cloudy days. Irradiation tests with monochromatic UV light at 300 and 307.5 nm showed the normal erythemal reaction. There was no amino-aciduria. Qualitative porphyrin screening tests were negative on stool and urine. Quantitative red cell porphyrin analysis was normal on two occasions (protoporphyrin 16 μg and 45 μg per 100 ml packed red cells).

When examined some time later, at the age of 22 mo the child showed definite signs of growth retardation, although he had had a normal birth weight (7 lb 6 oz) and had been delivered full term. He had grown, apparently normally, until the age of about 6 mo, but now seemed to have stopped. Apart from dwarfism no firm diagnosis is possible at the present time. The clinical picture does not fit xeroderma pigmentosum or Hartnup's syndrome. Cockayne's syndrome seems excluded because of the normal distribution of subcutaneous fat, the normal birth weight, the absence of ophthalmological defects, absence of ataxia and probably the presence of normal speech (Prof. I. A. Magnus and Dr. Philip Evans, personal communication). The karyotype was normal.

MATERIALS AND METHODS

Cell Culture

Cell strains used are shown in Table I. Fibroblast cultures were established by conventional techniques using plasma clots (cell strains 9257 and 11961) [12], or by a modification of this technique without plasma clots. Cultures were maintained in Eagle's minimal essential medium plus 15% fetal calf serum (Flow Laboratories, Irvine, Scotland). For experiments, cells were used between the 4th and 15th subculture with no detectable difference in results.

Cell Survival Experiments

The thin feeder layer technique of Cox and Masson [13] was used for plating (in 15% calf serum-Gibco-Biocult).

(a) For the response to 254 nm UV, 5 cm Nunclon plastic tissue culture grade dishes were inoculated with 2.4 x 10^6 cells in 4 ml of medium on day 1, and after removal of the medium they were irradiated with UV from an Hanovia low-pressure mercury lamp (type 15553) on day 2. The emission was predominantly at 254 nm and the fluence rate was as measured by a Latarjet meter was 0.96 W m⁻². For UV-sensitive strains the fluence was reduced by increasing the distance from the source and by the use of mask filters. Immediately following irradiation the cells were trypsinized (0.25% trypsin [Difco] in Dulbecco A buffer). After dispersal of the cells and resuspension in 2 ml of complete medium the cell number was determined and appropriate dilutions of cells dispensed on to the feeder layer in 10 ml fresh medium in 9 cm diameter dishes. The incubation period was 14–16 days. Colonies containing 50 or more cells were classified as survivors.

(b) Fluorescent sun lamp irradiation. Cells were handled in the same way as for 254 nm UV except that they were irradiated through the bottom of the dish on a rotating turntable 12 cm above a bank of 4 Westinghouse FS20 (20 w) sun lamps, whose emission maximum was at 310 nm. Spectrophotometric measurements indicated that all light below 285 nm was filtered out under these conditions.

(c) N-hydroxyacetamidinofochrome salt (N-hydroxy-AAF) 2.4 x 10^6 cells were incubated overnight in a 25 cm² Falcon 3013 flask in 4 ml medium. For treatment the N-hydroxy-AAF was dissolved at 2.5 mg/ml in Analar grade ethanol and then diluted in medium without serum to achieve the desired concentrations. The medium in the flask was then removed, the cells rinsed once with serum-free medium and incubated

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Table I. Cell strains

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Phenotype</th>
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<tbody>
<tr>
<td>9527°</td>
<td>Normal</td>
</tr>
<tr>
<td>1BR°</td>
<td>Sun-sensitive</td>
</tr>
<tr>
<td>2BI°</td>
<td>XP4LO°</td>
</tr>
<tr>
<td>11961°</td>
<td>XP-Excision-defective Group A*</td>
</tr>
<tr>
<td>XP4RO°</td>
<td>XP-Excision-defective Group C [42]</td>
</tr>
<tr>
<td>XP7TA°</td>
<td>XP Variants-normal excision, defective post-</td>
</tr>
<tr>
<td>XP3040°</td>
<td>replication repair [4]</td>
</tr>
</tbody>
</table>

* E. A. de Weerd-Kastelein and D. Bootsma - personal communication.

Cultures established from biopsies taken in our own laboratories.

Supplied by Dr. A. M. R. Taylor, Birmingham University.

Supplied by Drs. E. A. de Weerd-Kastelein and D. Bootsma, Erasmus University, Rotterdam.

at 37° C for 3 hr with the medium containing N-hydroxy-AAF. At the end of the treatment the medium was discarded and the cells rinsed once with the serum-free medium before trypsinization.

(d) Ethyl methanesulphonate (EMS) (Sigma). The cells were handled as for N-hydroxy-AAF except that the treatment was carried out in Dulbecco A buffer for 2 hr.

(e) Methyl methanesulphonate (MMS) (Sigma). The cells were handled as for N-hydroxy-AAF, except that treatment was carried out in Dulbecco A buffer with 500 μg/ml MMS for different periods of time.

(f) γ-Irradiation was carried out as described elsewhere [14]. Cells were exposed to 1 mCo γ-source at a dose-rate of approximately 2.4 krad/min.

 Unscheduled DNA Synthesis

(a) Autoradiographic procedures. 10° Cells were plated in 3.5 cm Petri dishes containing 2 coverslips. 15 hr later cells undergoing replication were differentially labeled with 10 μCi/ml of 3H-thymidine (24 Ci/mmol) for 1 hr. They were then washed with phosphate buffered saline (PBS), exposed to gemicidic UV light, and incubated in medium containing 10 μCi/ml of 3H-thymidine (12.5 Ci/mmol) for 1 hr in order to measure DNA repair synthesis. After washing with PBS the cells were fixed with 3% methanol acetic acid mixture. Autoradiographs were prepared using AR10 stripping film (Kodak) and an exposure time of 7-10 days.

(b) Scintillation counting. Cells were seeded at 2 x 10°/5 cm dish and grown for 7-10 days to form a confluent monolayer. The cells were treated with 10 mM-hydroxyurea for 30 min, then irradiated with UV light at 254 nm or from a fluorescent sun lamp and described in cell survival experiments. Medium containing 10 mM-hydroxyurea and 10 μCi/ml 3H-thymidine (20 Ci/mmol) was added and the cells incubated for 2 hr. The medium was then removed, the cells washed and scraped off the dishes into 0.3 ml 0.2 M NaOH. The viscous solution was sheared by stirring 5 times through the tip of a Fninipette and duplicate samples of 0.1 ml were taken onto Whatman 3MM paper squares (presoaked in 2% sodium dodecyl sulfate and dried). These were washed once in 5% trichloracetic acid/10 mM pyrophosphate, twice in 5% trichloracetic acid, once in ethanol, dried and the radioactivity counted in an Intertechnique liquid scintillation spectrometer. Plates cultured in parallel were trypsinized and the number of cells per dish was determined.

Excision of Pyrimidine Dimers

The procedure used was originally described by Paterson, Lohman, and Sluyter [15] and modified by Reynolds [16]. Briefly, UV-irradiated cells were incubated for different periods of time, the DNA extracted and treated with a crude extract of Micrococcus luteus which contained a "UV-specific endonuclease" activity. This activity introduces nicks in UV-irradiated DNA in the vicinity of pyrimidine dimers [15]. The details of the procedure as used in our laboratory have been described elsewhere [17].

RESULTS

(a) Cell Survival Experiments

The patient under study showed extreme sensitivity to sunlight at an early age, symptoms which might be expected if the child had XP. Fig 1A shows the response of several cell strains to the lethal effect of 254 nm UV (most of these data have been published previously in reference 18). It can be seen that cells derived from a skin biopsy of the patient at 9 mo of age showed a response to cell killing by 254 nm UV comparable with that of excision-defective XP cells. The response of cell strains from the parents was normal (results not shown). The UV sensitivity was confirmed with cells established from a second biopsy taken at 15 mo of age: these were slightly more sensitive than cells from the first biopsy.

The sensitivity of 11961 fibroblasts to light from a fluorescent sun lamp (emission maximum at 310 nm) was also enhanced like that of excision-defective XP cells (Fig 1B) and provides an explanation for the response of both XP and 11961 to natural sunlight, since wavelengths shorter than 290 nm are filtered out by the earth's atmosphere. It is presumably UV-lighe of wavelengths longer than 290 nm that actually inflicts the clinical damage [19].

Maher et al [7] have shown that cells from XP individuals exhibit an enhanced sensitivity to the activated carcinogen N-hydroxy-AAF. This hypersensitivity of XP cells was confirmed in our experiments but the cells of 11961 gave a normal response (Fig 1C). In contrast cells from 11961 were found to be hyper-sensitive to EMS, a mutagen to which the XP cells showed a

**Fig 1.** Cell killing in response to a number of DNA damaging agents. •••••, 1BR; □□□□, 2BI; ▼▼▼▼, XP7TA; ▼▼▼▼, XP3040. (A) UV (254 nm) irradiation. 1BR, 5 experiments, cloning efficiency (C.E.) 23-36%; 2BI, 5 experiments, C.E. 9-54%; XP7TA, 3 experiments, C.E. 7-9%; XP3040, 3 experiments, C.E. 17-25%; 11961 1 = first biopsy, 6 experiments, C.E. 12-96%; 11961 II = second biopsy, 3 experiments, C.E. 7-31%; XP4RO, 2 experiments, C.E. 19%; XP4LO 3 experiments, C.E. 16-72%. (B) UV (fluorescent sun lamp) irradiation. The dose is expressed as minutes of exposure. 1BR, 4 experiments, C.E. 17-96%; 2BI, single experiment, C.E. 75%; XP7TA, 3 experiments, C.E. 5-16%; XP3040, 2 experiments, C.E. 8-12%; 11961, 5 experiments, C.E. 2-17%; XP4RO, 3 experiments, C.E. 1-6%; XP4LO, 3 experiments, C.E. 2-28%. (C) N-hydroxy AAF exposure. 1BR, 2 experiments, C.E. 19%; 2BI 1 experiment, C.E. 43%; XP7TA, 1 experimental point, C.E. 28%; 11961, 3 experiments, C.E. 6-38%; XP4LO, 2 experiments, C.E. 6-20%. (D) EMS exposure. 1BR, 3 experiments, C.E. 11-43%; 2BI, 6 experiments, C.E. 2-35%; XP7TA, one experimental point, C.E. 23%; 11961, 3 experiments, C.E. 43-96%; XP4LO, 3 experiments, C.E. 8-10%.
normal response. Finally, neither 11961, nor the XP cell strains showed enhanced sensitivity to MMS (Fig 2A) or γ-rays (Fig 2B).

(b) DNA Repair After UV-Irradiation

The above experiments demonstrate that the cellular response of 11961 to DNA damaging agents showed some similarities and some differences to those of excision-defective XP cells. Further differences were revealed in studies of DNA repair after UV-irradiation. All XP cells tested have a defect in either excision-repair [3], postreplication repair [4] or both [18]. The technique of unscheduled DNA synthesis (UDS) is one method for measuring excision-repair in mammalian cells [20]. We have examined UDS in 2 different ways. The conventional autoradiographic procedure measures the ability of UV to stimulate non-S phase cells to synthesize small amounts of DNA. This unscheduled synthesis has been shown to be new DNA synthesized in gaps left after excision of DNA damage [21]. Table II shows that UDS was similar in normal and 11961 cells after irradiation at 254 nm.

We have also employed an alternative procedure using cells in stationary phase. If human fibroblasts were allowed to grow for several days, eventually a confluent monolayer was formed containing (5–12) × 10^5 cells per 5 cm dish. The small amount of residual DNA synthesis in these cells could be almost abolished by hydroxyurea [22]. Thus, under the conditions of our experiments during a 2 hr pulse with ^3H-thymidine only 100–300 cpm per 10^5 cells were incorporated into DNA. This was increased by UV-irradiation to about 2000–3000 cpm per 10^5 cells, presumably as a result of repair synthesis, which is known to be relatively insensitive to inhibition by hydroxyurea [23]. The UDS of 11961 cells, after irradiation with a 254 nm or a fluorescent sun lamp, was in the normal range (Table III). Although the variability in this type of experiment would preclude detection of a small reduction in UDS, the method demonstrated clearly the defect in UDS in the XP cell strain from complementation group A (Table III), in agreement with findings using other techniques.

An earlier step in excision-repair can be followed by measurement of the removal from the DNA of sites (presumed to be pyrimidine dimers) which are susceptible to attack by a damage-specific endonuclease present in extracts of *M. luteus* [15,24]. Such “dimer sites” are detected as a decrease in the molecular weight of the DNA strands after treatment with *M. luteus* extracts. ^3H-labeled normal cells and ^3H-labeled 11961 cells were UV-irradiated, incubated for various periods of time, and then mixed together. The DNA was purified, treated with extract of *M. luteus* and the molecular weight determined by sedimentation in alkaline sucrose gradients. The molecular weight distributions of the ^14C- and ^3H-labeled DNA were essentially superimposable under all conditions tested, indicating that loss of endonuclease-sensitive sites (pyrimidine dimers) occurred at equal rates in normal and 11961 cells. Processed data from such an experiment are shown in Fig 3. The small differences between the cell strains are well within experimental error. Similar results have been obtained independently by Dr. R. J. Reynolds (Medical Biological Laboratory, TNO, Rijswijk, Netherlands—personal communication). These experiments provide strong evidence that the number of pyrimidine dimers induced by UV in the DNA of 11961 cells is the same as in normal cells, and that the excision repair of these lesions is not defective in 11961 cells.

In a previous publication, we showed that postreplication repair in 11961 cells after UV irradiation was also normal [18]. There is thus a marked sensitivity for cell killing in these cells but no evidence of defects in either of the known pathways for the repair of DNA.

**DISCUSSION**

We have been able to show that, at the cellular level, the response of patient 11961 to a set of DNA damaging agents is quite different from that of XP cell strains (summarized in Table IV). The current clinical picture also shows no progression towards an XP condition. Cells from patients with Bloom's syndrome [25] have been shown to exhibit UV (254 nm) sensitivity both at the level of the individual [25] and the cell [26]. There are apparently no defects in excision [27] or postreplication repair [26]. Patient 11961, however, has neither the clinical nor the chromosomal [28] features of Bloom's syndrome. Preliminary reports have also indicated that cells from patients with Cockayne's syndrome [29] are also sensitive to UV (254 nm) [30,31]. At present, however, our patient does not fit this syndrome but the time scale for the development of the full clinical symptoms [29] does not yet permit a firm diagnosis. Should a positive clinical diagnosis for Cockayne's syndrome
ensue then clearly this case may represent another instance where cellular studies will have provided a first diagnosis.

The response of 11961 cells to the various DNA damaging agents is unusual. Activated derivatives of AAF produce bulky adducts principally at C-8 of guanine, and minor products at other positions on the purine bases [32]. The characteristics of excision repair in normal and XP cell strains after treatment with these derivatives has led to the suggestion that damage produced by this chemical carcinogen is repaired by the same mechanism as UV damage [33]. More recent experiments suggest that this idea is oversimplification. Thus Ahmed and Setlow [34] have shown that the rate-limiting steps for excision repair of damage induced by UV and N-acetoxy-AAF are different, since additive effects were observed at saturating doses. Furthermore Amacher, Elliott, and Lieberman [35] have shown that the characteristics of excision-repair of pyrimidine dimers and AAF adducts are markedly different. Our results showing that 11961 cells are hypersensitive to UV but not to N-hydroxy-AAF also suggest that the pathways which lead to recovery of cells from UV damage and AAF damage have some differences, although the defect in 11961 does not appear to lie in excision repair of pyrimidine dimers. The molecular basis for the difference in the response of 11961 to UV and N-hydroxy-AAF must await further clarification. (One possible, but unlikely explanation, could be that 11961 and XP cells have different capacities to convert N-hydroxy-AAF into its ultimate metabolite capable of reacting with DNA).

The hypersensitivity of 11961 cells to EMS but not to MMS was also unexpected. These mutagens alkylate the purine and to a lesser extent pyrimidine bases at various sites, in addition to producing phosphotriesters, apurinic sites and strand breaks [36]. The spectrum of alklylation products produced by EMS and MMS is, however, quite different, the former producing a greater proportion of 0-6 alkylguanines than the latter [36], so that the differential sensitivity to the 2 agents may not be quite as surprising as would appear. Moreover, the repair of damage produced by the alkylating agents, methyl- and ethyl- and both have been recently shown to have different characteristics [37].

The molecular basis for the sensitivity of 11961 cells to UV poses some novel questions about the recovery of cells from UV damage. There are several possible explanations:

(1) The defect lies in a recovery process unconnected with repair of DNA. Preliminary findings that 11961 cells are more mutable by UV light (our unpublished results), have a decreased capacity for host-cell reactivation of UV-irradiated adenovirus (R. S. Day, personal communication) and SV40 virus (P. A. Abrahams and A. van der Eb, personal communication) make this suggestion most unlikely. The work with SV40 virus cells which were transfected with SV40 DNA, strongly implicating DNA damage as the crucial factor leading to the hypersensitivity of 11961 cells.

(2) The cells are defective in excision-repair of pyrimidine dimers or in postreplication repair. It then becomes necessary to postulate that this defect could only be revealed by procedures with a greater sensitivity than those employed in our tests.

(3) The defect is in excision-repair of some other lesion induced by UV. Cells from XP variants, which have normal excision repair, have near normal UV sensitivity [5, 6, 8] despite having a defect in postreplication repair [4]. This suggests that the ability to excise damage is the major factor affecting cellular sensitivity to UV. UV light is known to cause other types of base damage apart from pyrimidine dimers, for example thymine glycols [38] and pyrimidine photohydrates [39]. There is to date, no good procedure for measuring these products when formed in DNA inside cells, nor are there any data assessing their biological importance in mammalian cells. It is conceivable that 11961 cells may have a defect in the excision of one of these types of nondimer photoproducts. If excision of this photoproduct normally uses the same enzymes as are involved in excision of damage induced by EMS, the sensitivity of 11961 cells to EMS could also be explained.

(4) The defect may lie in some other DNA repair process. XP cells, for example, are defective in photoreactivating enzyme [40] and an apurinic endonuclease [41]. 11961 may have similar defects. Alternatively his cells may be deficient in a repair process of whose existence we are at present totally unaware. Whichever explanation proves correct, 11961 cells can be tentatively regarded as another radiation-sensitive human mutant, which should be of value in elucidating the mechanisms of repair of radiation and other damage in human cells.

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