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Immunochemical determination of an initial step in thymine dimer excision repair in xeroderma pigmentosum variant fibroblasts and biopsy material from the normal population and patients with basal cell carcinoma and melanoma

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A monoclonal antibody specific for u.v.-induced thymine–thymine dimers in single-stranded DNA has been used in an enzyme immunoassay to investigate the loss of antigenicity associated with repair of this lesion in the first 2 h following 10 J/m^2 254 nm radiation. Variances of ±10% for the method and ±6.5% for individuals were established using primary cultures of biopsies from healthy individuals. No differences in the rate of loss of antigenicity was observed between 20 normal lymphocyte samples and 10 normal skin biopsies. Of three xeroderma pigmentosum (XP) variant cell lines tested, GM3617 could not be distinguished from normal cells but GM1227 and GM3053 showed lower rates of loss than any of the healthy samples. When the group mean values were compared there was no significant difference between normals and biopsies from sun-shielded skin areas from 16 basal cell carcinomas but similar material from 10 melanoma patients showed a significantly reduced ($P = 0.001$) rate of loss of antigenicity. Since the rate of loss of antigenicity in normal and XP variant cells reflected their relative abilities to perform unscheduled DNA synthesis, our results suggest that some melanoma patients may also have a minor deficiency in an early stage of excision repair.

Introduction

The recent development of sensitive enzyme immunoassays (EIA*) capable of quantifying low levels of specific types of DNA damage (1) has prompted their use for monitoring human environmental exposure to potential carcinogens (2–4) or for monitoring absorbed doses of chemotherapeutic agents (5). Another potential use of these assays is the screening of the human population for individuals having reduced DNA-repair capacity that may predispose them towards an increased risk of developing cancer. Such a relationship has been reported for u.v.-induced photoproducts in xeroderma pigmentosum (XP) cells defective in excision repair of thymine dimers (6,7).

In this paper we present the results of a study to test the feasibility of using an immunoassay to screen primary cell cultures for differential rates of the initial steps in thymine excision following 10 J/m^2 of u.v.-C radiation. An EIA was developed using a monoclonal antibody highly specific for the conformational change in single-stranded DNA (ss-DNA), caused by thymine–thymine dimers (8,9). The loss of antigenicity associated with the repair of dimers was investigated in groups of primary cultures derived from patients with XP variant syndrome, basal cell carcinoma (bcc) and melanoma, and compared with similar data obtained from lymphocytes and skin biopsies of normal healthy volunteers. The results demonstrate the ability of the assay to detect the minor impairments in photoproduct repair reported for some XP variants and melanomas. Basal cell carcinomas and melanomas are the most frequent skin tumors developed in XP variant patients and may be taken as an indication for heterozygote relatives (10).

*Abbreviations: EIA, enzyme immunoassays; XP, xeroderma pigmentosum; SS-DNA, single-stranded DNA; bcc, basal cell carcinoma; uds, unscheduled DNA synthesis.

Materials and methods

Chemicals

Adenine, calf thymus ss-DNA, polythymidilic acid, protamine sulphate grades II and X, protease and thymidine were obtained from Sigma. Phenol, pronase E, proteinase K, protease, ribonuclease, 1,2-phenylenediamine and thiomersal were obtained from Merck; skim milk powder from Fluka; fetal calf serum from Animed; MEM, vitamins, L-glutamine, non-essential amino acids and HBSS from Gibco; peroxidase-conjugated goat IgG raised against mouse IgG + IgM from Tago and Dulbecco's PBS 'A' tablets from Oxoid.

Cell culture and irradiation of cells

Three XP variant cell lines were obtained from NIGMS Human Genetic Mutant Cell Repository. Their DNA-repair capacity as determined by unscheduled DNA synthesis (uds) was 30–60, 56 and 88% that of normal controls for GM1227, GM3053 and GM3617 respectively (11).

Control samples were obtained from normal, healthy volunteers as 20 blood samples and 10 skin biopsies from 19 males and 11 females aged 25–57 years.

Biopsies from 16 basal cell carcinomas and 10 melanomas were supplied by the departments of Dermatology of the Kantonsspital, Basel and the University Hospital, Zürich respectively. All biopsies were taken from sun-shielded and non-malignant parts of the skin.

Monolayers. Biopsy samples were chopped and teased out under sterile conditions and explant cells were cultivated in MEM supplemented with 10% fetal calf serum, 1% non-essential amino acids, 2 mM L-glutamine and 2% vitamins. No antibiotics or antimycotics were added and the medium was changed every other day. After ~4 weeks the cultures were divided and grown in two 75-cm^2 flasks (Falcon Plastics) for an additional 2–3 weeks until confluent. The cultures were again trypsinized and the cells were divided into five Petri dishes (60 × 15 mm, Falcon) and cultured in fresh medium at 37°C and 5% CO_2 in a humidified incubator.

Table I. Effect of blocking mixtures on binding of antibody in EIA

Blocking mixture	ss-DNA unirradiated OD (490 nm)	u.v.-irradiated ss-DNA OD (490 nm)
1% thymidine	1.395 ± 0.078	2.592 ± 0.071
1% adenine	1.078 ± 0.010	2.615 ± 0.237
5% FCS	1.548 ± 0.073	2.445 ± 0.236
5% skim milk	0.695 ± 0.059	2.191 ± 0.153
2.5% FCS + 2.5% skim milk	0.780 ± 0.054	2.324 ± 0.093
5% FCS + 1% adenine	0.813 ± 0.119	2.324 ± 0.172
5% skim milk + 1% adenine	0.571 ± 0.012	2.133 ± 0.089
5% FCS + 1% thymidine	1.177 ± 0.043	2.348 ± 0.152
2.5% FCS + 2.5% skim milk + 1% adenine	0.748 ± 0.094	1.737 ± 0.173
2.5% FCS + 2.5% skim milk + 1% thymidine	1.308 ± 0.091	1.873 ± 0.153

PBSAT supplemented with the compounds shown was used as blocking agent in EIA (Materials and methods) with wells coated with 1 μg calf thymus ss-DNA that was unirradiated or irradiated with 10 J/m^2 u.v.-light. Values shown represent mean ± SE for groups of five wells tested.

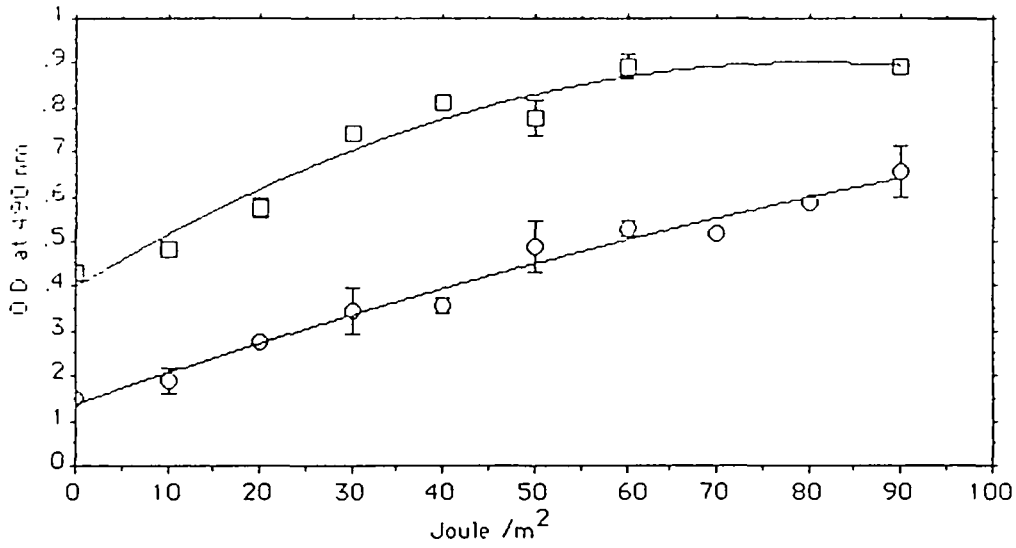

Fig. 1. Fluence dependency of EIA. Poly(dT) (○) and calf thymus ss-DNA (□) were irradiated with u.v.-C radiation and the increase in antigenicity caused by thymine dimer formation was assayed by EIA using triplicate samples. Points are mean values ± SD and curves are fitted by least squares.

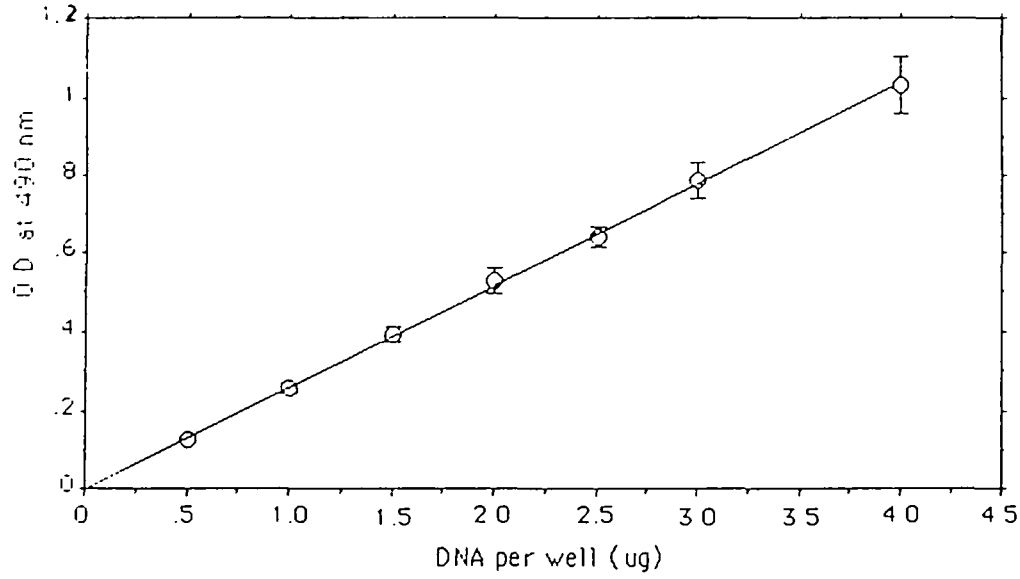

Fig. 2. Linearity of the antibody response (OD = 490 nm) with amount of DNA (μg). Each point represents the mean of five samples.

Blood cultures. Samples of 10 ml heparinized peripheral blood were centrifuged for 20 min at 300 *g*. Lymphocytes were isolated with a syringe, diluted 1:1 with prewarmed HBSS then centrifuged again on a Ficoll–Ronpacon gradient (d = 1.077, 300 *g*, 25 min) and washed twice with HBSS. The cells were resuspended in 10 ml culture medium and incubated for 4 h under the same conditions used for monolayers.

Irradiation. Irradiation was from a Phillips 6-V germicidal lamp emitting predominantly 253.7 nm radiation at 0.2 J/m^2/s at a distance of 38 cm. Each cell strain was divided into one non-irradiated and four irradiated aliquots. After pouring off the medium from the Petri dishes the samples were irradiated with 10 J/m^2. Fresh medium was added immediately and the cells were incubated at 37°C for 0, 10, 30 or 60 min. At each time point the repair processes were stopped by removing the medium and freezing the cells with liquid nitrogen.

Extraction and preparation of DNA

DNA was extracted by a phenol–ether method (12). The frozen cells were thawed and 1 ml of a solution which has the final concentration of 2% SDS, 0.1 mg/ml protease, 0.05 mg/ml proteinase K, 0.1 mg/ml pronase E and 0.1 mg/ml ribonuclease was added and the mixture was incubated at 37°C for between 6 h and overnight. One millilitre phenol saturated with buffer (0.1 M Tris–HCl, pH = 7.5, 0.75% hydroxyguanine) was added and vigorously shaken for 1 min, then slowly stirred for 10 min after which the mixture was centrifuged at 4000 *g* for 5 min and the aqueous phase was pipetted into a clean glass tube. After repeating

Table II. An example for the calculation of the loss of antigenicity measured by the bonding of the antibody, as observed in a healthy control (male, aged 38 years)

Time[a]	DNA (μg/ml)[b]	OD (490 nm)[c]	OD (μg/ml)[d]	Antigenicity[e]
0	12.0	0.378 ± 0.040	0.0315	100
10	15.5	0.336 ± 0.003	0.02	69
30	9.1	0.137 ± 0.007	0.0151	48
60	18.6	0.204 ± 0.026	0.0110	35
90	12.0	0.114 ± 0.019	0.0095	30
120	40.4	0.343 ± 0.055	0.0085	27
150	32.9	0.260 ± 0.009	0.0079	25

[a]Time points after u.v.-irradiation. 'O' was immediately following irradiation and the other times in minutes after u.v.-irradiation.
[b]Extracted amount of DNA as μg/ml of each sample.
[c]Mean of triplicate readings of the bound peroxidase-labelled antibody.
[d]The quotient of column (3) divided by column (2).
[e]The relative percentage of bound antibody/μg DNA using the 'O' time point as the 100% of antigenicity.

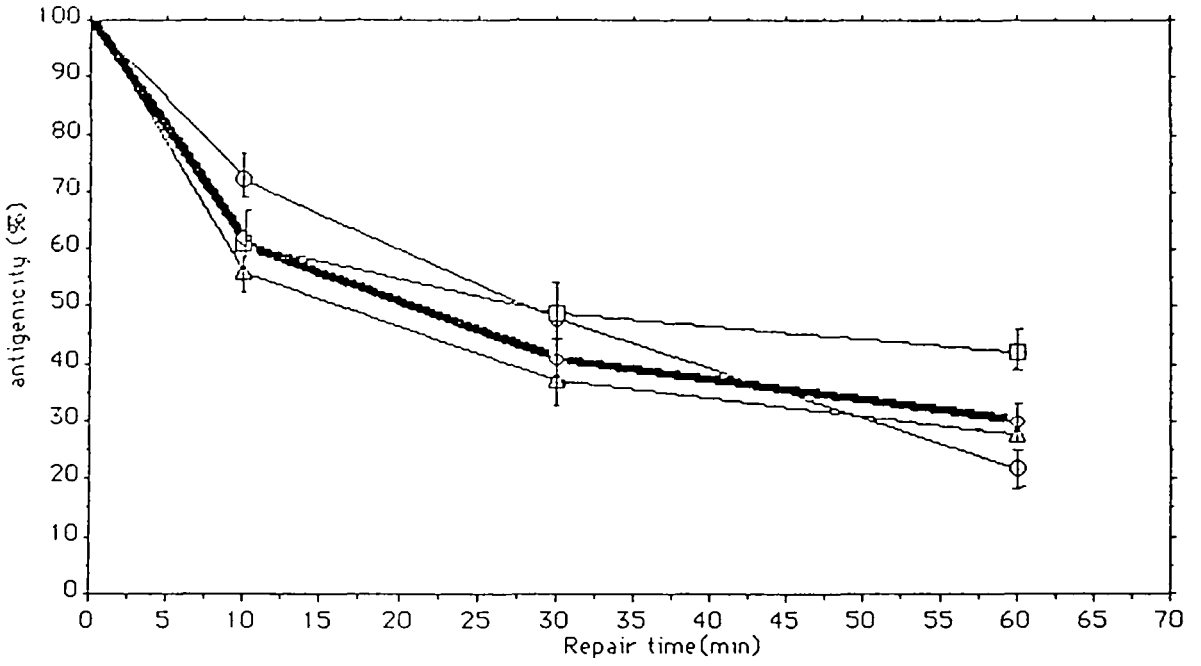

Fig. 3. The percentage loss of antigenicity in 30 normal cell lines after an u.v.-irradiation of 10 J/m^2. The dark line represents the mean ± SD of all 30 healthy individuals. To show the range of variance among healthy controls the response of three individuals (○, □ and △) are presented.

the phenol extraction the final aqueous phase was mixed with an equal volume of ether, shaken briefly, centrifuged and the ether layer discarded. This procedure was repeated until the aqueous phase was clear when a double volume of absolute ethanol was added. After mixing, the preparation was stored at −20°C overnight before centrifugation at 10 000 *g* in an Eppendorf centrifuge (Hettich). The pellet was dried and stored in a desiccator until used.

For quantitation, DNA was dissolved in 50 mM Tris−HCl, pH 7.5 containing 10 mM EDTA and 10 mM EGTA (13) and the optical density was measured at ~260 and ~280 nm. The ratio of the optical density at these wavelengths was reproducibly to 2.0 indicating that most protein had been removed. The amount of DNA was estimated by the formula: 1.0 OD unit at 260 nm = 50 μg/ml.

Enzyme immunoassay

EIAs were performed in 96 multiwell plates (Nunc) precoated with protamine sulphate to optimize the binding of DNA (13). To obtain ss-DNA, 0.5 ml of each sample was boiled for 10 min and quenched in ice water. Aliquots of 100 μl were added to the precoated wells and incubated overnight at 37°C. Non-adsorbed DNA was removed by five washes with blocking buffer (PBSAT containing 5% skim milk powder, 1% adenine). Non-specific binding sites were blocked by the addition of blocking buffer and incubation for 3 h at room temperature in the dark. After washing five times, the wells were incubated for 90 min at room temperature in the dark with anti-u.v. ss DNA-1 monoclonal antibody (8) diluted in blocking buffer, washed five times and incubated similarly with peroxidase-conjugated anti-mouse second antibody. Wells were washed five times with deionized water and incubated for 30 min at room temperature in the dark with 50 μl peroxidase reagent (40 mg 1,2-phenylenediamine in 100 ml 0.2 M citric acid, 0.1 M NaH_2PO_4, pH 5.0 to which was added 40 μl H_2O_2. The reaction was stopped after 30 min with 25 μl of 2.5 N H_2SO_4 and the optical density of each well was measured at 490 nm. Individual samples were assayed in triplicate unless otherwise specified.

Results

Establishing the EIA

Preliminary experiments showed high non-specific binding of antibody due to the suboptimal blocking conditions. Improved conditions were found by testing the binding of antibody to irradiated and non-irradiated ss-DNA after blocking with a variety of agents. The best was Dulbecco's PBS 'A' formulation plus 0.01% thiomersal (PBSAT) supplemented with 5% skim milk powder and 1% adenine (Table I), and it was used in subsequent experiments.

The linearity of the EIA as a function of u.v dose over the range 0−90 J/m^2 was examined using calf thymus ss-DNA and poly(dT) containing ~18−20 residues of thymidine per chain (Figure 1). A nearly linear response was obtained with poly(dT) over the whole range and the slope of the response is not significantly different to that of ss-DNA over the range 0−40 J/m^2. However, the displacement of the two response curves due to the greater binding of antibody to ss-DNA compared with poly(dT) in the absence of photoproducts is obviously evident. The standard deviation for poly(dT) was ±5.4% and for ss-DNA ±4.8%. A variance analysis with the Friedman test did not show any significant difference between the two antigens.

The response curves above clearly indicate that it is possible to use EIA to determine antigenicity of DNA in cells irradiated with 10 J/m^2, a dose known to kill ~15−20% of normal human fibroblasts (15). To simplify the handling of many different DNA samples it was necessary to demonstrate the linearity of the antibody response with the amount of DNA applied per well (Figure 2).

Table III. Loss of antigenicity in three XP variant cell lines

Cell lines	Incubation time (min)		
	10	30	60
GM1227	46[a]	55	54
GM3053	62	57	65
GM3617	84	79	84

[a]Values are percentage antigenicity lost compared with controls.

The loss of antigenicity in u.v.-irradiated normal human cells

Table II illustrates how the loss of antigenicity measured by the binding of the antibody in the DNA of a single individual is determined. For each time point three cell cultures were irradiated and each culture was carried out in triplicate. The standard deviation (SD) for the three independently repeated cultures of the same individual was ±6.5%. For the triplicate readings of bound antibody the standard deviation was ±10%; this gives the standard error of the method.

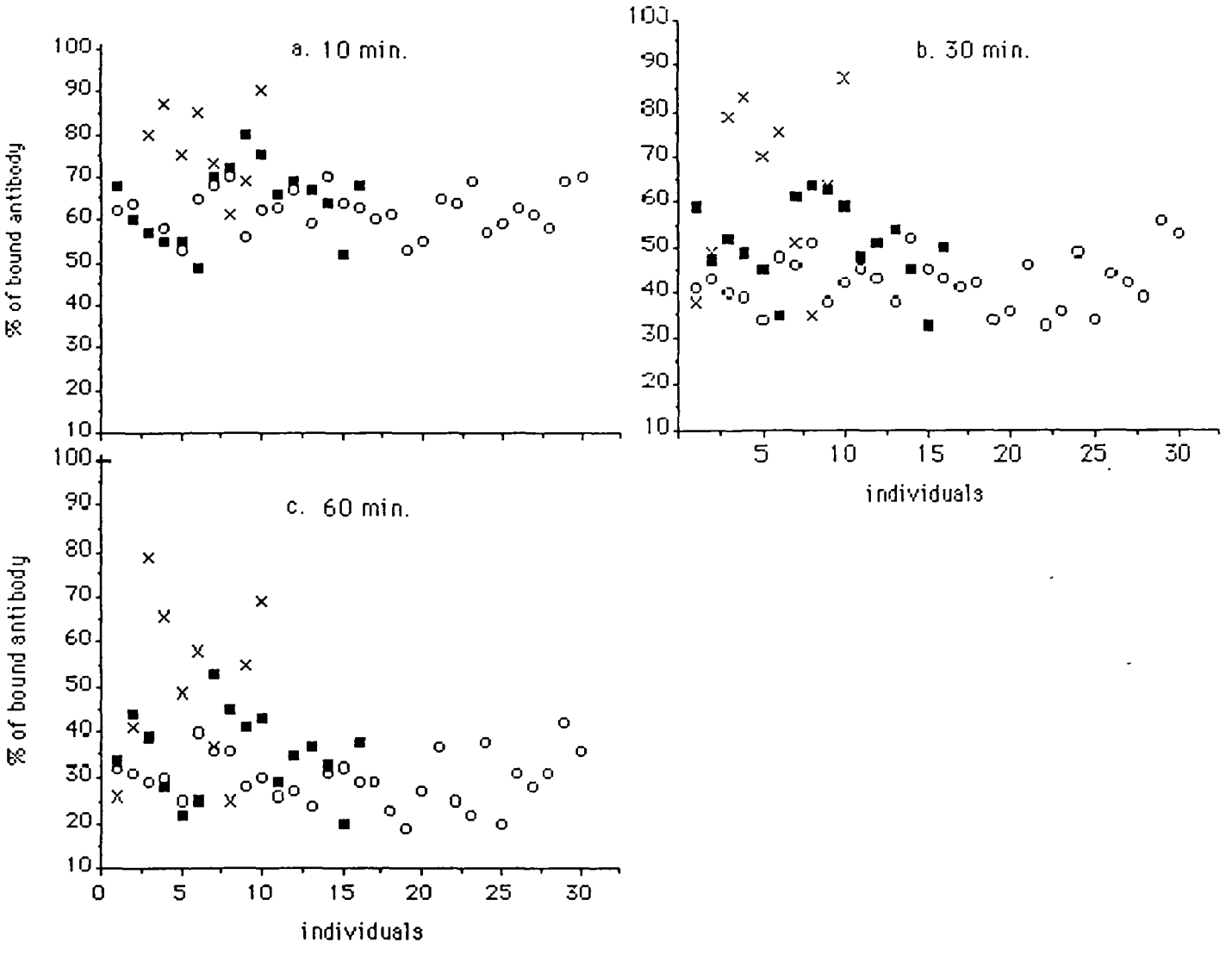

Fig. 4. Individual values of antigenicity at each time point, measured as % of bound antibody. Each individual is represented at the same point at all three time points (10, 30 and 60 min after u.v.-irradiation). O, Healthy controls; ■, bcc-patients; ×, melanoma patients. Melanoma cases 3, 4, 5, 6 and 10 showed an obvious higher amount of bound antibody. Among the bcc-groups 7, 8, 9 and 10 may belong to a subgroup with higher antigenicity, which would indicate a reduced loss of antigenicity, the calculation is shown in Table II.

The analysis of antigenicity included skin biopsies and lymphocytes from 30 healthy controls for whom the mean of the individual SD was ±8%. With these samples half of the specific antigenicity was lost during the first 30 min following irradiation (Figure 3). Figure 3 illustrates the variance in the normal response of three different individuals at 10, 30 and 60 min following irradiation. The mean for percentage of loss of antigenicity observed in normal controls at 10, 30 and 60 min after u.v.-irradiation are 62 ± 9%, 41 ± 12% and 30 ± 11.5% respectively (Figure 3).

The influence of disease states on loss of antigenicity

Cells from three XP variant patients showing reduced u.v.-light-induced uds with normal strand incision were tested for the loss of antigenicity (Table III). In comparison with the healthy controls GM1227 and GM3053 showed reduced rates of loss, whereas GM3617 is near the lower limits of the controls and would not have been identified as distinct from them.

These data are compared in Figures 4 and 5 with preliminary results obtained from cultivated fibroblasts of 16 patients with bcc and 10 with melanoma. Figure 4 represents the individual values of antigenicity at each time point for all the probands. Each individual is represented at the same point at all three time points. Figure 5 shows the kinetics of the loss of antigenicity as a function of time for all three groups and the three XP lines. Means, standard deviation, standard error and variance of each of the three groups are presented in Table IV for each time point. To distinguish whether the three groups show the same kinetics of antibody binding, an analysis of variance with the Mann–Whitney test (*U*-test) was performed. The results are presented in Table V.

Discussion

A number of syndromes that involve increased cancer risk have been shown to display abnormal cellular responses to u.v. light. Thus XP cells show hypersensitivity and altered repair of dimers (6,7,16–18) while altered repair synthesis is associated with mammary carcinoma (19,20) and Cockayne's syndrome shows hypersensitivity and altered RNA synthesis (21,22). Hypersensitivity to cell killing by u.v. is observed with some strains derived from Gardner's syndrome (23) and familial melanoma (24,25),

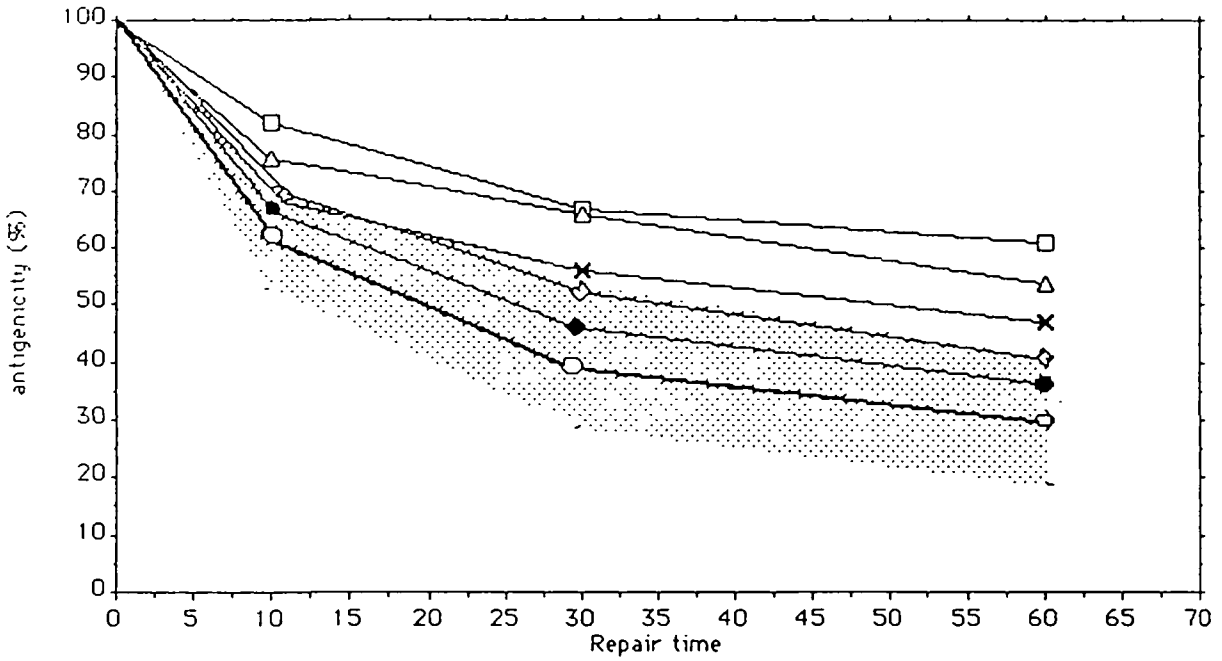

Fig. 5. Kinetics of loss of antigenicity in typical and atypical individuals. The extreme cases are found in the XP cell lines GM1227 (□) and GM3053 (Δ) followed by the melanoma patients, represented by the mean of 10 individuals (X). The kinetics of the third XP cell line GM3617 (◇) is within the normal kinetic range. The mean of the kinetics of the 16 bcc fibroblasts is represented by (●). The mean kinetic of the normal individuals is represented by the dark line (○) and the range is represented by the stippled area.

Table IV. Mean, standard deviation, standard error and variance for healthy, bcc and melanoma groups at each time point

Repair time (min)	Group	*n*	Mean[a] ± SD	SE	Variance
10	Healthy	30	62.17 ± 5.06	0.92	25.39
	Bcc	16	64.19 ± 8.73	2.16	76.16
	Melanoma	10	74.80 ± 10.56	3.34	111.51
30	Healthy	30	42.43 ± 5.98	1.09	35.70
	Bcc	16	50.93 ± 9.06	2.26	82.19
	Melanoma	10	63.1 ± 18.78	5.94	352.7
60	Healthy	30	29.80 ± 5.69	1.04	32.44
	Bcc	16	35.37 ± 9.00	2.25	81.05
	Melanoma	10	50.50 ± 18.24	5.77	332.94

[a]Values represent the mean of percentage of bound antibody.

Table V. Summary of the analysis of variance (Mann–Whitney *U*-test) for all three investigated patient groups tested one against the other

Combination of	Repair time (min)		
	10	30	60
Healthy : bcc	0.323	0.040	0.020
Healthy : melanoma	0.001	0.001	0.001
Bcc : melanoma	0.106	0.036	0.092

P values are shown for each time point and each combination of the three groups.

while cells of dysplastic naevus syndrome are hypermutable by u.v (26).

At present it is unclear what role reduced DNA repair plays in the development of malignancy in these diseases; whether it is the primary cause or merely an associated phenomenon (18,27).

Several immunoassays have recently been described which can measure dimers produced by fluences in the range 1–10 J/m^2 (8,28–35). In most cases the assays are of the competitive type. The assay we have developed is a solid-phase non-competitive assay similar to that described by Leipold *et al.* (32).

Using this assay we have observed, in agreement with others (28,29,34), a rapid loss of antigenicity that is faster than that reported for disappearance of dimers or endonuclease sites. The reason for this peculiar characteristic remains obscure, but because the antigen–antibody interaction of monoclonal antibodies is very precise we assume it implies a topological change at, or close to, the dimer site. The monoclonal antibody used does not seem to be specific for the dimer itself but for the conformational changes induced by the dimer as discussed by Strickland and Boyle (8) earlier. This possibility is supported by the findings of Paterson *et al.* (36) who suggested a new model for excision repair in humans. Whereas the pyrimidine dimer–DNA phosphodiesterase activity may be the earliest step in excision repair and causes a conformational change in the DNA before the classical repair steps (strand incision; lesion excision; patch insertion; strand ligation) take place.

Alternatively, the monoclonal antibody may be excluded by proteins of the repair complex that are possibly covalently linked to DNA. The loss of antigenicity is inhibited in excision defective XP cells in parallel with the inhibition of repair synthesis (30). Similarly we have observed a correlation between the percentage of uds reported for the three XP variant strains used here and the inhibition of the rate of loss of antigenicity. The comparative indices of antigenicity and the uds relative to normal control values are 51 ± 9.8% versus 30–60%, 61 ± 7.1% versus 56% and 82 ± 4.1% versus 88% for GM1227, GM3053 and GM3617 respectively. Evidently the loss of antigenicity is associated with an early step in excision rather than with post-replication repair, a process defective in all XP variant cells (37–39). We have investigated the kinetics of this early event during the first hour after irradiation of normal cells and unin-

volved cells from patients with melanoma and bcc.

Cells from a group of 30 healthy volunteers were used to define the range and mean of the normal response (Figure 3). Within this group different kinetic patterns were observed; their reproducibility and possible significance remain to be assessed. The group of 16 bcc patients did not differ significantly from the norm (Table V). Figure 4 could implicate a difference of some bcc patients from the others. We were unable to analyse the differences in more detail because there was no possibility of verifying the histological bcc categories (10) to which the patients were assigned. Individuals 7, 8, 9 and 10 have unique variances that may belong to a subgroup of bcc patients who will develop a bcc-related tumor in the forseeable future. Or they may have a genetic predisposition to one of those DNA-repair-deficient diseases.

Some of the 10 melanoma samples overlapped with the normal group (Figure 4), but the means of the two groups were significantly different, which suggest an abnormal response in some of the patients. Hypersensitivity has been reported in familial melanoma (25) and the present results suggest that this could be associated with defective DNA repair. Our findings did not enable us to use this test for diagnostic analysis. But in Figure 4, it is obvious that five of the melanoma patients (cases 3, 4, 5, 6 and 10) are different from all other investigated probands. This may be also due to the fact that we could not verify the differential diagnosis of each of the melanoma cases. Consequently we were unable to differentiate between familial, single and/or multiple melanomas.

By comparison of all three groups, in Table IV it is apparent that the normal controls showed the lowest variance, followed by the bcc and finally the melanoma patients. The summary of the statistical analysis is presented in Table V.

Thus the assay provides a useful non-isotopic method for screening of genetic mutations that effect cellular responses to u.v., particularly those involved early in excision repair. More precise analysis of additional data will involve the collection of a larger number of samples to enable age, sex, differential diagnoses, familial genetic predisposition for matching and confirmation of possible correlation with a reduction in the kinetics of the initial steps in thymine dimer excision repair. There was no significant difference between the results obtained with normal blood and skin samples suggesting that this assay is not grossly affected by heterogeneity of tissue types. A further consideration for future study is the influence of therapy regimes on the results obtained.

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