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Microinjection of *Micrococcus luteus* UV-endonuclease restores UV-induced unscheduled DNA synthesis in cells of 9 xeroderma pigmentosum complementation groups

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Summary

The UV-induced unscheduled DNA synthesis (UDS) in cultured cells of excision-deficient xeroderma pigmentosum (XP) complementation groups A through I was assayed after injection of *Micrococcus luteus* UV-endonuclease using glass microneedles. In all complementation groups a restoration of the UV-induced UDS, in some cells to the repair-proficient human level, was observed. Another prokaryotic DNA-repair enzyme, T4 endonuclease V, restored the UV-induced UDS in a similar way after microinjection into XP cells. Since both enzymes specifically catalyse only the incision of UV-irradiated DNA, we conclude that this activity is impaired in cells of all 9 excision-deficient XP complementation groups tested.

The autosomal recessive human disorder xeroderma pigmentosum (XP) is characterized by an extreme sensitivity of the skin to sunlight, predisposition to skin cancer and frequently neurological abnormalities (see Kraemer, 1983 for a review). Cultured fibroblasts of most XP patients are deficient in the removal of ultraviolet light (UV) induced pyrimidine dimers from their DNA and show a decreased rate of unscheduled DNA synthesis (UDS) (Cleaver, 1968; Bootsma et al., 1970). Using cell fusion techniques, these excision-deficient XP cells have been classified into 9 genetically distinct complementation groups, designated A through I (Kleijer et al., 1973; de Weerd-Kastelein et al., 1974; Kraemer et al., 1975; Arase et al., 1979; Keijzer et al., 1979; Moshell et al., 1983; Fischer et al., 1985). This abundance of mutually complementary mutations suggests a considerable genetic (and biochemical) complexity of the process in which UV-induced pyrimidine dimers are removed from mammalian DNA. So far, no genes or gene products involved in XP have been isolated.

Tanaka et al. (1975, 1977) have reported that the UDS in fibroblasts of XP complementation groups A through E can be restored to the level of normal human cells by the introduction of T4 endonuclease V using cell-permeabilization by Sendai virus. Using the same technique, Hayakawa et al. (1981) subsequently found restoration of UV-induced UDS in XP-F group cells. However, in this case correction was less than 50% of the

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wild-type level. Since T4 endonuclease V is known to catalyse the first step (incision) of the prokaryotic DNA-repair process (Yasuda and Sekiguchi, 1970; Minton et al., 1975), these results support the conclusion that the excision-deficient XP cells used are deficient in the incision of UV-damaged DNA. Cells of XP complementation groups G–I have so far not been investigated in this way.

We have recently used microneedle injection of crude human cell extracts into cultured XP fibroblasts to identify proteins in these extracts that are able to restore the UV-induced UDS of the injected cells (de Jonge et al., 1983; Hoeijmakers et al., 1983). Using the same technique we have also studied the effect exerted by various non-human DNA-repair enzymes on the UV-induced UDS in excision-deficient XP cells. This report concerns the restoration of UV-induced UDS in XP cells representative of XP complementation groups A through I by microinjection of *Micrococcus luteus* UV-endonuclease. We also report here that T4 endonuclease V introduced into excision-deficient XP cells by microinjection can restore the UV-induced UDS of XP complementation groups C through G, thus confirming and extending the results of Tanaka et al. (1975) and Hayakawa and coworkers (1981).

Materials and methods

Information on the XP cell lines used in this study is given in Table 1. Cells were cultured in Ham's F10 medium (Flow) with 7.5% foetal calf serum (FCS), 7.5% newborn calf serum, 100 IU penicillin and 100 μg streptomycin per ml. Some fibroblast strains were grown in the same medium containing 15% FCS. Details of the microinjection procedure and subsequent assay for UV-induced UDS have been described previously (de Jonge et al., 1983). In short, homopolykaryons of each XP cell line were generated by cell fusion with the aid of β-propiolactone-inactivated Sendai virus (de Weerd-Kastelein et al., 1972) and cultured for at least 3 days to allow completion of DNA replication (S-phase) before they were used for microinjection. For each experiment enzyme was injected into the cytoplasm of at least 50 homopolykaryons (2–8 nuclei per cell) using glass microneedles according to the procedure of Graessmann et al. (1980). Relevant data on the injection and injected cells were recorded during microinjection with the aid of a tape recorder.

The assay for UV-induced UDS was performed by UV-irradiation (20 J/m^2), culture in the presence of [^{3}H]thymidine and visualization of the radioactivity incorporated into repair patches of the DNA by autoradiography. Either assay procedure A, performed as described (de Jonge et al., 1983) was used, or a more sensitive procedure (procedure B) which differed from procedure A on the following points: the culture medium contained dialysed foetal calf serum (15%), [^{3}H]-thymidine of high specific activity (46–80 Ci/mM) and fluorodeoxyuridine (1 μM). These modifications result in a 6-fold increase in sensitivity.

TABLE 1

RELEVANT INFORMATION ON THE CELL LINES USED

Cell line designation	XP complementation group	Relevant characteristics	Reference
XP25RO	A	Excision deficient	Kraemer et al. (1975)
XP11BE	B	Excision deficient	Kraemer et al. (1975)
XP21RO	C	Excision deficient	Kleijer et al. (1973)
XP1BR	D	Excision deficient	W. Keijzer (unpublished)
XP2RO	E	Excision deficient	De Weerd-Kastelein et al. (1974)
XP126LO	F	Excision deficient	W. Keijzer (unpublished)
XP2BI	G	Excision deficient	Keijzer et al. (1979)
XPCS2	H	Excision deficient	Moshell et al. (1983)
XP3MA	I	Excision deficient	E. Fischer et al. (1985)
C5RO	–	Normal human	

The level of UV-induced UDS was calculated from the average number of silver grains per nucleus (±SEM) determined for the polynucleated cells that survived the microinjection treatment (usually more than 70% of the injected cells). For comparison, the level of UDS was also determined for a population of noninjected homopolykaryons on the same slide. The observed level of UDS was related to the UV-induced UDS of repair-proficient C5RO fibroblasts assayed in each experiment as a standard. The wild-type UDS level varied between experiments but was always higher than 50 grains per nucleus. The use of homopolykaryons has the advantage that confusion of grains due to UDS with grains due to a short period of S-phase incorporation of [^{3}H]thymidine is avoided because the nuclei of polykaryons no longer enter S-phase 3 or more days after fusion (Jaspers et al., 1981; unpublished observations).

M. luteus UV-endonuclease, corresponding with fraction II in the purification method of Carrier and Setlow (1970), was generously provided by Drs. G. van der Schans and L. Roza (TNO, Rijswijk). T4 endonuclease V, purified and stored as described (Seawell et al., 1981), was a generous gift from Dr. A.A. van Zeeland (State University, Leiden). Immediately before use, the T4 enzyme preparation was dialysed for 30 min against 1000 vol. of reversed phosphate-buffered saline (RPBS, 4.05 mM Na_2HPO_4; 1.1 mM KH_2PO_4; 140 mM KCl; pH 7.2) in order to remove ethylene glycol (which proved to be lethal to the injected cells).

Results

Homopolykaryons of XP complementation groups A through I were assayed for UV-induced UDS after microinjection of *M. luteus* UV-endonuclease in the cytoplasm. Quantitative data from these experiments are presented in Table 2. For all complementation groups the microinjected cells displayed a higher mean grain count than noninjected neighbouring homopolykaryons although the degree of stimulation varied between different complementation groups. The grain count also varied between different experiments with the same cells (cf. Tables 2 and 3) as well as between individual homopolykaryons injected in the same experiment (data not shown). This variation probably reflects differences in the amount of enzyme injected.

TABLE 2

LEVELS OF UV-INDUCED UDS IN HOMOPOLYKARYONS AFTER MICROINJECTION OF *M. luteus* UV-ENDONUCLEASE

Cell line [a]		UDS [b] (grains per nucleus) as % of wild type ± SEM		
		Non-injected	Injected	
C5RO		103 ± 4	100	(A)
XP25RO	(A)	1 ± 1	79 ± 4	(B)
XP11BE	(B)	9 ± 1	54 ± 3	(B)
XP21RO	(C)	20 ± 1	37 ± 3	(A)
XP1BR	(D)	17 ± 1	55 ± 2	(B)
XP2RO	(E)	50 ± 2	65 ± 5	(A)
XP126LO	(F)	19 ± 1	50 ± 2	(B)
XP2BI	(G)	2 ± 1	23 ± 2	(A)
XPCS2	(H)	36 ± 2	86 ± 4	(B)
XP3MA	(I)	14 ± 1	55 ± 3	(B)

[a] In parentheses, the XP complementation group.

[b] The UDS procedure used (see Materials and Methods for details) is given between brackets.

Fig. 1 shows a trinucleated XPCS2 cell (XP complementation group H) injected with *M. luteus* UV-endonuclease, together with 3 noninjected mononucleated XPCS2 fibroblasts (one of which is in S-phase) after UV-irradiation and the autora-

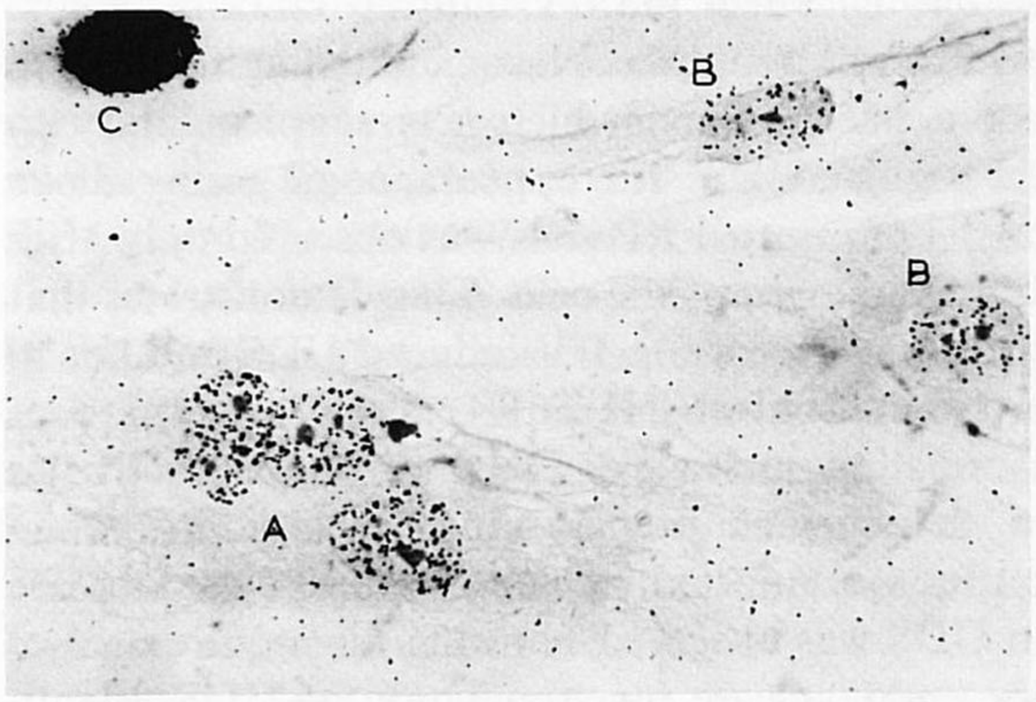

Fig. 1. Photomicrograph of a XPCS2 homopolykaryon (containing 3 nuclei) after microinjection of *M. luteus* UV-endonuclease assay for UDS and autoradiography. See Materials and Methods for experimental details. A, injected homo trikaryon; B, noninjected monokaryons; C, monokaryon in S-phase.

TABLE 3

LEVELS OF UDS IN HOMOPOLYKARYONS AFTER DIFFERENT TREATMENTS

Cell line [a]		Microinjection	UV-irradiation	UDS [b] (grains per nucleus) as % of wild-type ± SEM
C5RO		–	+	100
C5RO		*M. luteus* UV-endonuclease	+	101 ± 4
XP25RO	(A)	–	+	2 ± 1
XP25RO	(A)	RPBS [c]	+	2 ± 1
XP25RO	(A)	*M. luteus* UV-endonuclease	–	1 ± 1
XP25RO	(A)	*M. luteus* UV-endonuclease	+	56 ± 6
XP2BI	(G)	–	+	2 ± 1
XP2BI	(G)	*M. luteus* UV-endonuclease	–	1 ± 1
XP2BI	(G)	*M. luteus* UV-endonuclease	+	39 ± 4

[a] The XP complementation group is given in parentheses.

[b] Assay procedure A was used.

[c] RPBS, reversed phosphate-buffered saline, see Materials and Methods.

diographic assay for UDS. Many autoradiographic grains can be seen above the nuclei of the injected tri-karyon, whereas considerably fewer grains are observed over the nuclei of the noninjected cells. For the tri-karyon shown, the average grain count per nucleus was 95% of the grain count of repair-proficient C5RO cells used as a standard in this experiment.

In a series of experiments carried out with cells from XP complementation groups A and G the specificity of the observed increase in grain count was investigated. As shown in Table 3, microinjection of *M. luteus* UV-endonuclease did not noticeably affect the UV-induced UDS in repair-proficient C5RO fibroblasts, indicating that this is not a phenomenon which acts supplementary to the regular UDS. The appearance of grains above nuclei of injected XP cells was observed only after UV-irradiation of the cells. This demonstrates that the grains are due to UV-induced UDS and not to incorporation of ^{3}H-TdR caused e.g. by contaminating nuclease or DNA polymerase activities in the enzyme preparation. Furthermore, when buffer was injected instead of enzyme no increase in UDS was observed, showing the requirement of the endonuclease for stimulation of UDS. Identical results were obtained with mononucleated and polynucleated cells (data not shown) and the number of nuclei in an injected homopolykaryon did not have a noticeable effect on the level of UDS reached since the mean grain counts for cells with

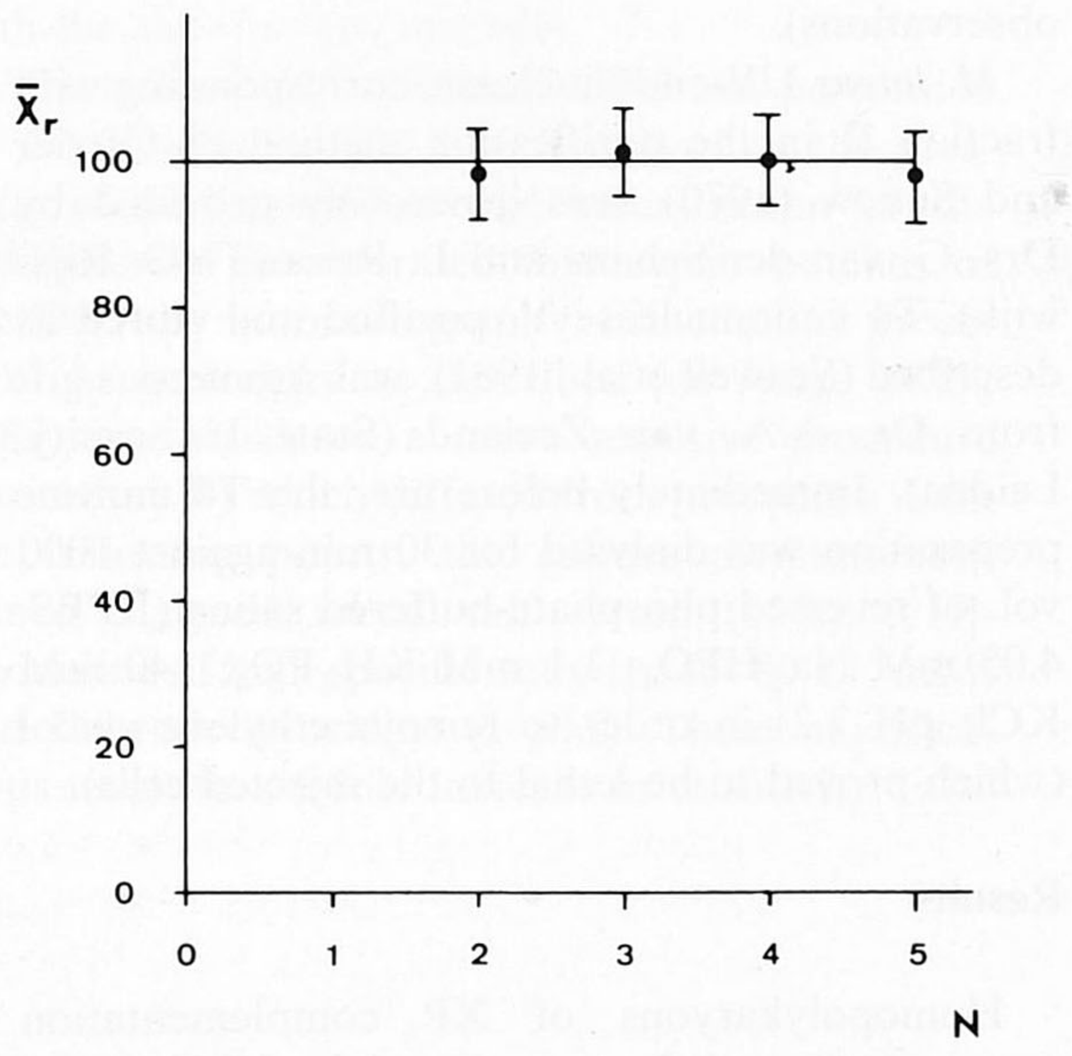

Fig. 2. The level of UV-induced UDS in relation to the number of nuclei in microinjected homopolykaryons. The grain count of an injected homopolykaryon was expressed as a percentage of the average grain count calculated for the total population, to obtain the relative grain count, X_r. Subsequently, a classification was made according to the number of nuclei per cell (N) and for each category (bi-, tri-, tetra- and poly-karyons) the mean of the relative grain counts, $\bar{X}_r$, was determined ± SEM. This method of calculation was followed because the results of different experiments (with different values for the average grain count of the total population) were combined in order to obtain sufficient cells of each category (bikaryons, 53 cells; trikaryons, 32 cells; tetrakaryons, 14 cells and polykaryons, 25 cells).

TABLE 4

LEVELS OF UV-INDUCED UDS IN XP HOMOPOLYKARYONS AFTER MICROINJECTION OF T4 ENDONUCLEASE V

Cell line [a]		UDS [b] (grains per nucleus) as % of wild type ± SEM	
		Non-injected	Injected
XP25RO	(A) [c]	2 ± 1	23 ± 1
XP25RO	(A) [c,d]	2 ± 1	18 ± 2
XP21RO	(C)	17 ± 1	31 ± 1
XP1BR	(D)	28 ± 1	39 ± 2
XP2RO	(E)	50 ± 2	65 ± 3
XP126LO	(F)	17 ± 1	61 ± 3
XP2BI	(G)	3 ± 1	56 ± 2

[a] In parentheses, the XP complementation group.

[b] Unless stated otherwise assay procedure A was used (see Materials and Methods).

[c] UDS assay according to procedure B (see Materials and Methods).

[d] T4 endonuclease preparation 1:1 diluted with injection medium.

2, 3, 4 or 5 and more nuclei did not differ significantly from the mean of the total population (Fig. 2). These experiments demonstrate that the UV-induced UDS in excision-deficient XP fibroblasts can be restored by microinjection of *M. luteus* UV-endonuclease.

As shown in Table 4, T4 endonuclease V was also able to restore the UV-induced UDS after microinjection into homopolykaryons of the XP complementation groups tested. As was found with *M. luteus* UV-endonuclease, a number of individual cells was complemented to wild-type UDS level but the average number of grains was lower than the wild-type level.

To investigate whether the level of correction of UDS is influenced by the amount of enzyme injected the T4 endonuclease preparation was diluted 1:1 with injection medium and injected into XP25RO (XP-A) fibroblasts. As shown in Table 4 the mean number of grains reached using the diluted enzyme is lower than that observed with the nondiluted preparation.

Discussion

The experiments presented here demonstrate a correction of the excision-repair deficiency in cells of all XP complementation groups after microinjection of the prokaryotic DNA-repair enzyme *Micrococcus luteus* UV-endonuclease and of T4 endonuclease V in the cases tested. These results confirm and extend the findings of Tanaka et al. (1975, 1977) and Hayakawa et al. (1981). These authors reported a restoration of UV-induced UDS in XP cells of groups A through F after introduction of T4 endonuclease V into XP cells permeabilized by concomitant treatment with Sendai virus. The correction of XP cells of group F was only to 50% of the wild-type (wt) UDS in spite of the fact that the amount of T4 enzyme used was 3-fold in excess of the concentration required for the correction of XP group A cells to the wt level (Hayakawa et al., 1981).

In our experiments the average level of UV-induced UDS varied between cell strains and to some extent also between experiments with the same cells. Usually UDS was restored to less than the level of normal human cells, although a number of individual injected cells did display wt grain numbers. When the results of Tanaka et al. (1975) are taken into account it seems probable that – at least in the case of XP-cells of groups A through E – limiting amounts of enzyme have been injected into most of the cells. This is supported by the finding that dilution of the T4 endonuclease preparation yielded a lower level of UDS in XP-A fibroblasts than the non-diluted sample (Table 4). Although quantitative comparison of UDS levels obtained after microinjection is difficult, due to the fact that the injected volume is not constant, this finding suggests that in the case of T4 endonuclease the amount of enzyme is at least one of the factors that limit the level of correction.

With cells of group F, UDS levels were obtained comparable to those found by Hayakawa et al. (1981). It has to be established whether the UDS found in XP cells of groups G through I was limited by the amount of enzyme injected or whether the maximally obtainable level of correction was reached in those cells.

As mentioned by Hayakawa et al. (1981) in relation to XP group F cells, a possible explanation for a correction to less than the wt UDS level could be that not all lesions are readily accessible to the exogenous enzyme, e.g. because the lesions are masked by an inactive protein or repair com-

plex. The work of Mortelmans et al. (1976) and Kano and Fujiwara (1983) suggests that the defect in cells of some XP complementation groups may reside in an altered chromatin structure. It is possible that in those cases the injected prokaryotic endonucleases are not able to reach some of the dimers during the assay period, despite their small size of only 16–18 kD (Nakabeppu and Sekiguchi, 1981; Grafstrom et al., 1982).

The fact that cells of all 9 excision-deficient XP complementation groups are corrected at least partly by enzymes that incise DNA near pyrimidine dimers suggests that the defect in all these XP cell strains resides at stages before or at the incision step of the DNA-excision-repair pathway. If the number of complementation groups in XP actually reflects the number of polypeptides required in these early stages of repair, it is noteworthy that a function which involves at least 9 polypeptides in human cells can be performed by a prokaryotic enzyme on its own. However, in this respect it should be taken into account that the microbial endonucleases used here have only pyrimidine dimers as a substrate (Friedberg et al., 1981) whereas the excision-repair process in human cells acts on a much broader spectrum of DNA lesions (Grossman, 1981).

Both *M. luteus* UV-endonuclease and T4 endonuclease V catalyse the incision of UV-irradiated DNA in a two-step reaction: the pyrimidine dimer is attacked successively by a dimer–DNA glycosylase which hydrolyses the 5′-glycosyl bond of the dimer and by an apyrimidinic endonuclease which cleaves the phosphodiester backbone 3′ of the apyrimidinic site (Haseltine et al., 1980; Nakabeppu and Sekiguchi, 1981; Grafstrom et al., 1982).

It has not been established whether one of these activities alone is sufficient for the correction of all XP excision-repair defects, or whether the concerted action is required. The mechanism of action of the equivalent human UV-endonuclease is not known. It is possible that the normal human enzyme yields reaction products different from the microbial enzymes we have used, as has been found for the uvrA, B, C complex of *Escherichia coli* (Sancar and Rupp, 1983). Therefore, the UDS observed in excision deficient XP cells treated with *M. luteus* UV-endonuclease or T4 endonuclease V could reflect a DNA-repair pathway involving steps not normally followed during excision repair in human cells.

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