Characteristics of excision repair of pyrimidine dimers in eukaryotic cells as assayed with anti-dimer sera

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ABSTRACT
An antiserum that recognizes the three types of pyrimidine dimers induced by UV-light in DNA was used to monitor their removal from eukaryotic cell nuclei. Cells able to excise dimers display a similar removal pattern: a fast process completed within a few hours leaving many dimers in the DNA.

INTRODUCTION
Antisera that recognize cyclobutane type of pyrimidine dimers (UV-DNA sera) have been obtained in several laboratories by immunizing rabbits with UV-irradiated DNA (1-6), and were used to follow the elimination of dimers from the DNA of bacteria and eukaryotic cells (3,6,7). Although pyrimidine dimers are the most prominent lesions induced in DNA irradiated with 254 nm UV-light, minor products are formed (8) that might be antigenic for the immunized animals. If UV-DNA sera are used to determine the presence of pyrimidine dimers in DNA, for instance to study excision repair, the certainty must exist that only these products are bound by the sera. Therefore, the UV-DNA sera have to be analysed for the presence of antibodies that might fix other DNA alterations than dimers.

We have attempted to continue the characterization of UV-DNA sera. Furthermore, the removal of antibody fixing capacity was determined in UV-irradiated cells from eukaryotic origin known for their capability to eliminate pyrimidine dimers in order to gain information about UV-DNA sera and in the hope of being able to explain discrepancies between this technique and other methods that measure directly pyrimidine dimers in the DNA. Moreover, excision was also studied in yet untested cells.

MATERIAL AND METHODS
Cell culture. African green monkey kidney cells (CV-1; CCL-70), diploid syrian hamster embryo fibroblasts, Chinese hamster cells (V79), bat lung cells (Tb-1,
from Taradia brasiliensis), secondary chick embryo cells and human diploid fibroblasts were grown in Petri dishes in thymidine free medium supplemented with 9% newborn calf serum and 1% foetal calf serum.  

**Irradiation procedures.** Ultraviolet radiation was delivered from a germicidal lamp giving predominantly 254 nm light. The absorbed dose was measured with a Latarjet dosimeter. Photoreactivating light was given at 37°C as flashes from two xenon filled lamps as described previously(5).  

**Affinity columns.** Bovine serum albumin(BSA) was coupled to sepharose 4B (Pharmacia, Uppsala) according to Poonian et al(9). Calf thymus DNA from Worthington was purified by chloroform–isoamyl alcohol(24:1) extraction. The DNA was denatured with 0.1 M NaOH for 10 min at 37°C. Single-strand DNA (SS-DNA), UV-irradiated(5000 J/m²) or not, was linked to sepharose–BSA with carbodiimide according to Rickwood(10). γ-globulin fractions were obtained by(NH₄)₂SO₄ precipitation of UV-DNA sera and subsequent DEAE-cellulose fractionation, resulting in almost pure IgG preparations. The columns were loaded with these γ-globulin fractions in phosphate buffered saline (PBS) and washed with buffer until the absorbance of the effluent was less than 0.01 at 280 nm. When as much material was recovered from the column as was applied, it was considered saturated. After saturation, antibodies were eluted with 0.5 M acetic acid, neutralized immediately with solid K₂HPO₄ and dialysed against PBS.  

**Dimer analysis.** Cells grown on coverslips in Petri dishes were fixed in acetic acid–ethanol(1:3). The coverslips were mounted on glass slides and passed for 2 min through 0.07 M NaOH in 70% ethanol in order to denature the DNA. The slides were rinsed with water, dried, and stored at -20°C prior to use. The relative number of pyrimidine dimers in nuclear DNA was determined by autoradiography with tritiated γ-globulin fractions eluted from affinity columns. Tritiated immunoglobulins(7)(45 μg/ml; 10⁶ dpm/μg protein) in PBS in the presence of 5% BSA were incubated for 30 min with the cells. After extensive rinsing in PBS that contained 1% BSA, in PBS and in running tap water, the slides were processed for autoradiography in Ilford K₂ or L₄ nuclear emulsions. Grains over at least 50 nuclei per slide were counted.  

**Radioimmune assays.** The activity of IgG fractions towards UV-irradiated DNA and the relative number of photoproducts in DNA were determined with competitive radioimmune assays(5). Percent inhibition was calculated as described(5). Purified single-stranded calf thymus DNA was irradiated with ionizing radiation from a ⁶⁰Co-gamma source(Gammacell 220, Atomic Energy of Canada Ltd) in the presence of sufficient oxygen. Oxidation of DNA with OsO₄
at neutral pH was performed as described by Hariharan and Cerutti(11). Oligo dC was a product from Worthington.

RESULTS

It has previously been shown that UV-DNA sera bind thymine containing pyrimidine dimers in DNA structures(5). As cytosine dimers in oligo dC are also recognized by the serum(TABLE I )it may be assumed that the three possible pyrimidine dimers that can be produced in DNA are bound by the UV-DNA sera. If DNA is irradiated with γ-rays or treated with the oxidizing reagent OsO₄ , damage of the so called 5,6-dihydroxy-dihydrothymine type (t¹ type) is introduced in the thymines in high amounts(11), whereas UV-irradiation gives only limited numbers of these lesions(12). Compared with untreated single-stranded DNA neither γ-ray irradiated DNA nor DNA treated with the oxidant competed with the UV-irradiated DNA for binding to the immunoglobulin(TABLE I ),which makes it unlikely that DNA lesions of the t¹ type are recognized by UV-serum.

Since UV-DNA sera possess affinity for single-stranded DNA(3,5,6), we attempted to purify and concentrate the active antibodies.IgG was absorbed in DNA-sepharose columns and the eluants and eluates tested for activity towards UV-DNA.Sepharose columns containing single-stranded DNA did not retain anti dimer antibodies because all the active proteins passed the

<table>
<thead>
<tr>
<th>inhibitor</th>
<th>% binding</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>oligo dC (0.1 μg)</td>
<td>71.5</td>
<td></td>
</tr>
<tr>
<td>UV-oligo dC (0.1 μg)</td>
<td>78.7</td>
<td></td>
</tr>
<tr>
<td>SS-DNA (7.4 μg)</td>
<td>37.1</td>
<td>51.9</td>
</tr>
<tr>
<td>SS-DNA (14.7 μg)</td>
<td>70.4</td>
<td>1.5</td>
</tr>
<tr>
<td>SS-DNA (29.4 μg)</td>
<td>68.9</td>
<td>3.6</td>
</tr>
<tr>
<td>γ-SS-DNA (7.4 μg)</td>
<td>62.0</td>
<td>13.0</td>
</tr>
<tr>
<td>γ-SS-DNA (14.7 μg)</td>
<td>71.3</td>
<td>0.3</td>
</tr>
<tr>
<td>γ-SS-DNA (29.4 μg)</td>
<td>70.3</td>
<td>3.5</td>
</tr>
<tr>
<td>OsO₄-SS-DNA (4.8 μg)</td>
<td>63.6</td>
<td>11.1</td>
</tr>
<tr>
<td>OsO₄-SS-DNA (9.6 μg)</td>
<td>71.1</td>
<td>0.6</td>
</tr>
<tr>
<td>OsO₄-SS-DNA (19.2 μg)</td>
<td>66.7</td>
<td>6.7</td>
</tr>
</tbody>
</table>

radioimmunoassay was performed with 3 μg UV-irradiated single-stranded DNA (15000 dpm), 200 μl antiserum and 40 μl inhibitor. γ-SS-DNA: 20 Krad. UV-oligo dC: 5000 J/m² UV-light.
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The UV-DNA serum can apparently not be freed from residual activity for unirradiated DNA. This implies that a UV-DNA serum does not contain a mixture of antibodies against single-stranded DNA and dimer containing DNA, but that the antibodies of the IgG population that recognize dimers possess low activity for single-stranded DNA as well.

Columns containing UV-irradiated single-stranded DNA, however, retained all the active antibodies applied (TABLE II). The effluents from these columns had UV-DNA binding properties only after saturation, whereas the eluates were about 4-5 times more active than the γ-globulins initially used (Fig. 1).

When active IgG was applied over a column containing only BSA coupled to sepharose, no protein binding could be detected. The efficiency of elution varied considerably from experiment to experiment and ranged from 10-45% of the proteins stuck. Since low dissociation constants are generally to be expected with polyvalent antigen systems (13), anti UV-DNA antibodies with the highest affinity for UV-DNA remained probably bound to the UV-DNA on the matrix.

Excision of dimers

Excision of dimers was determined by autoradiography with radiolabelled antibodies eluted from UV-DNA affinity columns that fixed UV-irradiated DNA in cell nuclei. The reliability of the technique has been proven by comparing the immunologic method with the direct analysis of pyrimidine dimers in DNA extracted from UV-irradiated cells by paper chromatography (7). With both methods the dimers disappeared from the DNA of irradiated HeLa cells to the same extent and with similar rates.

TABLE II Activity of anti UV-DNA immunoglobulin passed through DNA affinity columns.

<table>
<thead>
<tr>
<th>inhibitor added</th>
<th>% binding</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>original γ-globulin</td>
<td>84.4</td>
<td>21.8</td>
</tr>
<tr>
<td>effluent SS-DNA sepharose column 1</td>
<td>67.6</td>
<td>19.8</td>
</tr>
<tr>
<td>effluent SS-DNA sepharose column 2</td>
<td>65.1</td>
<td>22.8</td>
</tr>
<tr>
<td>effluent UV-SS-DNA column 1</td>
<td>84.0</td>
<td>0.5</td>
</tr>
<tr>
<td>effluent UV-SS-DNA column 2</td>
<td>85.2</td>
<td>0</td>
</tr>
<tr>
<td>eluate UV-SS-DNA sepharose column 1</td>
<td>22.4</td>
<td>73.4</td>
</tr>
<tr>
<td>eluate UV-SS-DNA sepharose column 2</td>
<td>27.3</td>
<td>67.7</td>
</tr>
</tbody>
</table>

Radioimmune assay was performed with 3 μg UV-SS-DNA (2888 dpm tritium), 200 μl antiserum and 20 μl inhibitor protein in PBS (40 μg protein).
Monkey cells exhibited similar rates of excision after various low UV-doses (Fig. 2). The removal of dimers was fast and terminated about 3-5 h after irradiation when approximately half of the induced lesions were eliminated. No further loss of dimers was detected even 60 h after irradiation suggesting that a longer mitotic delay follows after a single UV-treatment in these cells than in human or Chinese hamster cells. The monkey cells removed the same percentage of dimers when irradiated between 5 and 20 J/m² UV-light. It suggests that independent of the exposing dose about half of the dimers are induced in inaccessible positions for repair enzymes.

Similar excision data were obtained with HeLa cells using the same technique. The results are in agreement with those obtained by others with monkey cells concerning the final 50% excision but this value was only reached gradually at non saturating UV-exposures.

A similar rate and extent of dimer removal as found in monkey cells was detected in diploid human fibroblasts. Secondary chick embryo cells were capable of performing both excision repair of dimers and photoreactivation (Fig. 3). The excision process is less active in these cells than in human or monkey cells as only 30% of the dimers were eliminated during 5 h post-irradiation incubation in the dark. The decline of the dimer content with time in the dark can totally or partly account for the repair replication and unscheduled synthesis that was observed in these cells after UV. By the combined action of photoreactivation and excision, however,

Fig. 1. Antidimer activity of a γ-globulin fraction eluted from a UV-DNA-sepharose column. 78% of 3μl UV-SS-DNA (19100 dpm) was fixed to 200 μl antiserum in the absence of inhibitor. 25 μl inhibitor protein in PBS was added, original γ-globulin fraction (•); eluate (○).
Fig. 2. Removal of pyrimidine dimers from UV-irradiated monkey cells. Cells were irradiated with UV-light and incubated in the dark. -○- and -□-, 5 J/m²; -■-, 15 J/m²; -▲-, 20 J/m². The autoradiographic exposure time in L₄ emulsion was 10 days.

Fig. 3. Removal of dimers from chick and human fibroblasts. Cells were irradiated with 20 J/m² UV-light and incubated in the dark or in the presence of photoreactivating light. -○-, chick cells exposed to light; -▲-, chick cells incubated in dark; -■-, human cells incubated in dark after UV. The autoradiographic exposure time was 14 days in L₄ emulsion.
almost 90% of the dimers were eliminated from the nuclei within 2 h incubation in medium after UV. Very similar results for experiments done in light were presented by Paterson et al. (17) who used an UV-endonuclease technique for assessing dimers. These authors, however, detected only a limited removal of dimers in the dark (17).

Bat cells are also able to excise dimers from the DNA with similar efficiency as cells of primate origin (Fig. 4). Neither Chinese hamster V79 cells from which removal of dimers could not be shown by means of chromatography (18, 19), nor Syrian hamster cells showed any sign of dimer excision (Fig. 4).

Since the eventual presence of photoreactivation capacity can hardly be detected in cells possessing an active dimer excision mechanism because of the rapid photoproduct removal, photoreactivation of the dimers was tried in Syrian hamster cells. No decrease with time in grain number over nuclei that had been illuminated with visible light after UV-exposure.

Fig. 4. Removal of dimers from the DNA of Chinese hamster, bat, and Syrian hamster cells. After irradiation cells were incubated in dark or in the presence of photoreactivating light. Chinese hamster cells (■) irradiated with 10 J/m² (26 days exposure in K₂ emulsion), Syrian hamster cells irradiated with 10 J/m² and incubated in the dark (○) or in the presence of visible light flashes (●), exposure time 12 days in L₄. Bat cells (▲) irradiated with 30 J/m² UV-light grown in dark, 10 days exposure in K₂ emulsion.
could be observed (Fig. 4). In view of the fact that the presence of the photoreactivating enzyme, at least in human diploid fibroblasts might depend on the composition of the culture medium (20), the results do not definitively mean that these rodent cells are photoreactivation deficient. Similarly, photoreactivation of dimers was undetectable in excision-deficient mouse epidermal cells in vivo (21).

DISCUSSION

Since γ-globulin fractions from UV–DNA sera cannot be freed from a low activity for single-stranded DNA, it is believed that this property of the sera is a direct result from the procedure followed to obtain anti-dimer sera, i.e., the immunisation of rabbits with UV-irradiated DNA. As a consequence, the anti-dimer antibodies recognize only pyrimidine dimers in DNA structures (5) and might even need the presence of one or more adjacent unaltered bases on one or both sides of the dimer as in the hapten for appropriate binding to occur as is strongly suggested by results from radio immune assays with short UV-irradiated oligodeoxythymidines (1, 6).

As has been pointed out by Seaman et al. (6), these adjacent bases could add to the stability of the antibody receptor-antigenic determinant complex. It means that there is a low built-in affinity for unirradiated DNA that cannot be separated from the anti-dimer activity. This property might be common to sera obtained after immunization of animals with polynucleotides containing a certain amount of altered bases.

The fast early loss of dimers after irradiation found in the excision proficient cells could not be detected in human cell lines (22). The number of thymine in dimers per unit dose obtained by these authors with a UV-lamp that emits principally at 254 nm was relatively high compared with other data from human or in general mammalian cells (7, 16, 23, 24, 25). The authors might therefore, have used doses which saturate the excision repair system, a situation which was carefully avoided in the experiments presented in this report.

It has been speculated that cells from animals active during daytime should be able to remove dimers whereas cells from nocturnal animals might be excision deficient (26). Rat cells, however, were capable of removing pyrimidine dimers efficiently.

All types of cells so far assayed for excision of dimers with labelled antibodies exhibited a similar pattern of dimer removal in the dark; the entire process completed within a few hours with a fast early loss of
dimer but leaving many dimers in the DNA(5,7). These data are in perfect agreement with the time courses of repair synthesis generally obtained in UV-irradiated human(2,27) and rodent cells(19,27,28). For dimer excision deficient cells, as Chinese hamster V79 for instance, the results imply that repair synthesis after UV-exposure does not reflect dimer removal but the elimination of yet unknown photoproducts.

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