DNA Repair in V-79 Cells Treated with Combinations of Ultraviolet Radiation and \(N\)-Acetoxy-2-acetylaminofluorene\(^1\)

Farid E. Ahmed and R. B. Setlow

Biology Department, Brookhaven National Laboratory, Upton, New York 11973

SUMMARY

Earlier experiments on human cells showed that \(N\)-acetoxy-2-acetylaminofluorene mimics ultraviolet radiation in biological and repair characteristics and that the amount of repair from a combined treatment was additive. Chinese hamster V-79 cells are less proficient than human cells in excision repair of pyrimidine dimers resulting from irradiation. We therefore investigated the combined effects of both xero-derma pigmentosum pigments. Normal human fibroblasts are able to repair DNA damaged by UV radiation and AAAF\(^2\) by excision repair involving long patches (~100 nucleotides) (17), whereas conventional xero-derma pigmentosum cells lack these abilities (6, 24). Moreover, xero-derma pigmentosum cells are more sensitive than normal cells, by approximately the same factor, to the cytotoxic and mutagenic activity of both agents (15). We showed earlier that in normal human fibroblasts the amount of repair resulting from a combined treatment was additive and that AAAF treatment did not inhibit the removal of sites sensitive to an exogenous UV endonuclease (1). Chinese hamster cells have a much lower capacity to perform excision repair of UV damage than human fibroblasts (22). The excision of thymine dimers is difficult to demonstrate in hamster cells by radiochromatography (8, 25, 26), but V-79 cells perform nearly as much repair replication as normal human cells at early times after irradiation (5). Hence, we wished to determine the ability of these cells to perform excision repair of AAAF damage and the amount of repair as a result of a combined treatment with UV and AAAF. We used 3 techniques: (a) unscheduled DNA synthesis measured radioautographically (7), (b) the photolysis of BrdUrd incorporated into parental DNA during repair (18), and (c) the loss of sites sensitive to a UV endonuclease (16). The 1st 2 techniques measure repair due to both agents, whereas the 3rd allows us to detect only the pyrimidine dimers caused by UV. Radioautographic data showed that the amount of unscheduled DNA synthesis due to AAAF treatment was between one-half and two-thirds that due to UV. Moreover, the extent of repair after a combined treatment was much less than the sum of the 2 treatments separately, and at high concentrations of AAAF the amount of unscheduled DNA synthesis in a combined treatment was less than that due to UV or AAAF alone. BrdUrd photolysis studies confirmed these results and showed that repair of AAAF damage in V-79 cells was of the long-patch type (approximately 130 nucleotides). The measurements of UV-endonuclease-sensitive sites showed that 10 \(\mu\)M AAAF resulted in an approximately 55% inhibition of the removal of sites resulting from a dose of 3.6 J/sq m UV.

INTRODUCTION

Normal human fibroblasts are able to repair DNA damaged by UV radiation and AAAF\(^2\) by excision repair involving long patches (~100 nucleotides) (17), whereas conventional xero-derma pigmentosum cells lack these abilities (6, 24). Moreover, xero-derma pigmentosum cells are more sensitive than normal cells, by approximately the same factor, to the cytotoxic and mutagenic activity of both agents (15). We showed earlier that in normal human fibroblasts the amount of repair resulting from a combined treatment was additive and that AAAF treatment did not inhibit the removal of sites sensitive to an exogenous UV endonuclease (1).

\(^1\) This research was carried out under the auspices of the United States Energy Research and Development Administration. By acceptance of this article, the publishers and/or recipient acknowledges the U.S. government's right to retain a nonexclusive, royalty-free license in and to any copyright covering this paper.

\(^2\) The abbreviations used are: AAAF, \(N\)-acetoxy-2-acetylaminofluorene; BrdUrd, 5-bromo-1-deoxyuridine; dThd, deoxythymidine; \(M_n\), weight-average molecular weight; \(M_n\), number-average molecular weight.

MATERIALS AND METHODS

Cell Culture. Chinese hamster cells, V-79, were obtained from M. Bender, Medical Department, Brookhaven National Laboratory, Upton, N. Y. They were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, l-glutamine (400 \(\mu\)g/ml), penicillin (140 units/ml), and streptomycin (140 \(\mu\)g/ml) (Grand Island Biological Co., Grand Island, N. Y.) and were kept in a humidified 10% CO\(_2\) atmosphere at 37°.

Unscheduled DNA Synthesis. Twenty-four hr before treatment, cells were seeded at a density of \(10^4\) cells/sq cm onto 11-x 22-mm coverslips (Arthur H. Thomas Co., Philadelphia, Pa.), contained in glass Petri dishes, 60 mm in diameter, and covered with 5 ml of medium. One hr before treatment, hydroxyurea (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 200 \(\mu\)g/ml.
DNA Repair in V-79 Cells Treated with UV Plus AAAF

Mo.) was added to a final concentration of 4 mM to inhibit scheduled DNA synthesis (7). At zero time (time of treatment) the cells in growth medium were exposed for 20 min to various concentrations of AAAF (a gift from J. A. Miller), dissolved in fresh (CH$_3$)$_2$SO (Fisher Chemical Co., Springfield, N. J.) or the medium was removed and the cells exposed to various doses of 254-nm radiation at a dose rate of 0.36 watt/sq m, or to a combined treatment of UV followed by AAAF, or AAAF followed by UV. After treatment, fresh medium containing 4 mM hydroxyurea and [H$^3$]thymidine, 5 µCi/ml (6.6 Ci/mmole; New England Nuclear, Boston, Mass.), was added and left for 1 hr. The coverslips were then removed, fixed in Carnoy’s solution, hydrated in a descending series of alcohols, and immersed in distilled water. Cells were stained by the Feulgen procedure (9), and the coverslips were dipped in 2-fold-diluted Kodak NTB photographic emulsion and kept in the dark for 4 days at 4°. They were developed in Kodak D-19 developer and fixed in a Kodak fixer. Fifty cells were selected randomly, and the number of silver grains per nucleus was counted. The appropriate backgrounds (8.2 to 11.2 grains/nucleus) for hydroxyurea without UV irradiation or hydroxyurea with (CH$_3$)$_2$SO equivalent to that used in AAAF treatment (13 to 52 mm) were subtracted.

Bromodeoxyuridine Photolysis. The general details of the technique have been described previously (17, 18). We used 2 plates of cells per assay, each labeled with a different radioactive precursor. Approximately 500,000 cells were plated in 5 ml of medium in 60-mm plastic dishes. One plate was labeled for 24 hr with [H$^3$]thymidine, 1 µCi/ml, and the 2nd plate was labeled for 24 hr with [C$^14$]thymidine, 0.4 µCi/ml (50 Ci/mole; New England Nuclear).

After the labeling period, the medium was discarded, and the cells in both plates were treated for 20 min with various concentrations of AAAF, or cells were exposed to various doses of 254-nm radiation or to a combined treatment of UV followed by AAAF. Immediately after treatment, cells were incubated in a fresh medium containing 2 mM hydroxyurea and 0.1 mM BrdUrd (Sigma Chemical Co.) for cells labeled with [H$^3$]thymidine or containing 2 mM hydroxyurea and 0.1 mM dThd (Sigma Chemical Co.) added to cells labeled with [C$^{14}$]thymidine. Both plates of cells were incubated at 37° for 12 hr to allow repair and incorporation of BrdUrd or dThd to take place. They were then washed with phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 2.0 g Na$_2$HPO$_4$, and 0.4 g H$_2$PO$_4$ in 1 liter distilled H$_2$O, pH 7.2) and exposed to 2000 R of X-rays to facilitate strand separation in the subsequent alkaline sedimentation (10), and the cells were harvested and mixed together at 10$^4$ cells/ml in cold EDTA-0.8% NaCl solution (23). These cells were exposed for times up to 8 hr to wavelengths of light above 305 nm to selectively photolyze the BrdUrd incorporated into parental DNA during repair (12). The photolytic light was obtained from a water-cooled Philips SP 500-watt high-pressure mercury arc lamp in which unfiltered light was focused through a thin layer of Mylar [to absorb wavelengths less than 305 nm (14)] onto a quartz microcuvet containing 0.2 ml of cells. Fifty thousand cells were lysed on top of an alkaline sucrose gradient (5 to 20%, 2 ml NaCl), and the DNA was centrifuged at 30,000 rpm for 150 to 170 min in either an SW 50.1 or SW 60 rotor. Fractions were collected from the bottom of the gradient, and the acid-insoluble radioactivity was counted and converted to average molecular weight by a computer program as described elsewhere (18). The amounts of radioactivity per gradient were between 10,000 and 13,000 cpm for each isotope.

The $M_u$'s of cells incubated in dThd and BrdUrd were obtained for different photolytic doses (see below). The difference between their reciprocals $\Delta(1/M_u) = (1/M_u)_{\text{BrdUrd}} - (1/M_u)_{\text{dThd}}$ is a measure of the photolysis of BrdUrd incorporated into parental DNA during repair.

Photolytic Dose. Since the light source emits a broad range of wavelengths, most of which are ineffective in photolysis (20), measurement of dose in J/sq m is useless. Hence, we estimated the dose in terms of the number of breaks per BrdUrd residue that would be made by exposure to the light source. The latter estimation was made from measurements of the numbers of breaks per dalton, $(1/M_u)$, in DNA fully substituted with BrdUrd, as a function of exposure time, by sedimentation in alkaline as described above. In actual practice we used such determinations of breaks to calibrate a YSI-Kettering Model 65 radiometer that measured the light passing through a 313 nm band pass, 12.4 nm full-width, one-half-maximal filter (Oriel G-521-3130, Oriel Corp. of America, Stamford, Conn.). This filter-radiometer combination gives a good estimate of the relative effective dose rate, since shorter wavelengths are absorbed by the Mylar, and longer ones are ineffective in photolysis. A meter reading of 12 watts/sq m corresponded to 4.6 x 10⁻³ breaks per BrdUrd per min and approximately 0.4 x 10⁻⁸ breaks/dalton/min in unsubstituted DNA.

Endonuclease-sensitive Sites. Details of the assay have been described before (16, 19). DNA containing dimers is exposed to an extract from Micrococcus luteus, and the UV endonuclease in the extract makes single-strand breaks, the number of which equals the number of pyrimidine dimers (21). The amount of excision in hamster cells is small. To achieve more sensitivity and minimize experimental fluctuations, we compared, in a single assay, DNA from cells labeled with different isotopes and representing untreated and treated cells, treated unincubated and treated incubated, or UV-irradiated and UV-plus-AAAF-treated cells. Approximately 100,000 cells were plated in 5 ml of medium in 60-mm plastic dishes. One plate was labeled for 24 hr with [H$^3$]thymidine, 0.4 µCi/ml, and the 2nd plate was labeled for 24 hr with [C$^{14}$]thymidine, 0.4 µCi/ml. The labeled cells were treated, and at the end of the treatment or incubation period the cells in each plate were washed with an ice-cold EDTA-containing NaCl solution before they were rubbed off into 2 ml of the same medium, mixed together, and centrifuged. The pellet was dispersed, and the cells were lysed in 0.5 ml of lysing solution containing 0.02 µM Tris-HCl, pH 8, 0.04 µM NaCl, 0.002 µM EDTA, and 10% Sarkosyl (Ciba Geigy Corporation, Ardsley, N. Y.). Pronase (Calbiochem, La Jolla, Calif.) was added to 25 µg/ml, and the proteins were digested for 60 min at 37°. The DNA was extracted with 1 ml of phenol equilibrated with endonuclease buffer (0.02 µM Tris-HCl, pH 8, 0.04 µM NaCl, 0.02 µM EDTA) by gentle rotation for 1 hr. The aqueous layer containing DNA was collected and extracted 2 times with an equal
F. E. Ahmed and R. B. Setlow

volume of ether saturated with endonuclease buffer, and
the DNA solution was dialyzed at 4° overnight against 2
changes of buffer. The endonuclease used was a crude
extract equivalent to Fraction III of Carrier and Setlow (3).
Five µl of the extract (5 mg protein per ml) added to 100 µl
of DNA solution at 37° for 20 min were sufficient to take the
enzymatic reaction to completion. The reaction was termi-
nated by removing the mixture with a wide-tipped micropipet
and layering it on top of a 5 to 20% alkaline sucrose
gradient containing 0.5 M NaCl, with 0.2 ml of 1 M NaOH
layered on its top, and the DNA was sedimented at 20° in an
SW 60 rotor of a Beckman L5-50 ultracentrifuge at 50,000
rpm for 75 min. Fractions were collected, the acid-insoluble
radioactivity was counted in a scintillation counter, and the
distribution of counts was converted to average molecular
weights by a computer program as described previously.
The amounts of radioactivity per gradient were between
4,000 and 6,000 cpm for each isotope.

We used $M_u$ rather than $M_s$ since the latter is very sensi-
tive to fluctuations in the amount of radioactivity near the
top of a gradient. We assumed that the breaks were distrib-
uted randomly and took $M_u = M_s/2$. The number of breaks
per unit molecular weight is then $1/M_s$, and the change in
$1/M_s$ as a result of endonuclease treatment represents the
number of endonuclease-sensitive sites per dalton. The
 nonspecific endonucleolytic activity as measured on unirra-
diated DNA was less than 0.1/10^6 daltons, and in each
experiment controls receiving the same treatment but with-
out endonuclease addition were used to correct for any
nonspecific breaks or slightly different molecular weights of
the extracted DNA. The latter numbers were less than 0.2/
10^6 daltons.

RESULTS

Unscheduled DNA Synthesis. (CH₃)₂SO at the highest
concentration used (52 mM) resulted in a slight increase of
grain count above background (11 compared to 8 grains/
nucleus) and did not affect unscheduled synthesis resulting
from UV. The results of the autoradiographic studies on the
effects of various doses of UV, or AAAF, or a combined
treatment of both, are shown in Charts 1 and 2. Unsched-
uled synthesis approaches saturation at a UV dose of 20 J/
sq m and an AAAF concentration of 20 µM. However, at the
saturation level AAAF results in approximately two-thirds of

![Chart 2. Relationship between unscheduled DNA synthesis, expressed as
number of grains per nucleus, and various AAAF concentrations. •, AAAF
alone; O, AAAF + 5 J/sq m UV; □, AAAF + 10 J/sq m UV; △, AAAF + 20 J/sq
m.](chart2)

![Chart 3. Alkaline sucrose gradients of DNA from V-79 cells irradiated with
10 J/sq m UV, incubated for 12 hr in nonradioactive BrdUrd (BrdU) or dThd,
and then exposed to a photolytic dose of 2.9 x 10^-2 breaks/BrdUrd residue. Values
of $M_u$ were 30.3 x 10^6 for cells incubated in BrdUrd (O) and 58 x 10^6 for cells
incubated in dThd (△). S, sedimentation.](chart3)

the unscheduled DNA synthesis resulting from UV. More-
over, the level of unscheduled DNA synthesis after a com-
bined treatment is less than additive and at high doses and
concentrations the amount of unscheduled DNA synthesis
in a combined treatment is less than that from UV or AAAF
alone. It is apparent from Chart 2 that AAAF treatment
inhibits unscheduled synthesis resulting from UV irradiation
and that UV inhibits unscheduled synthesis arising from
AAAF treatment. Similar results were obtained (data not
shown) when AAAF was added immediately after UV radia-
tion and left for the entire period of incubation (1 hr), or
when cells were pretreated with AAF for 20 min followed
by UV irradiation.

Bromodeoxyuridine Photolysis. Chart 3 shows sedimen-
tation profiles of labeled cells irradiated with 10 J/sq m of
UV radiation, then incubated for 12 hr in nonradioactive
BrdUrd or dThd, and exposed to a photolytic dose of 2.9 x
10^-2 breaks/BrdUrd residue. $\Delta(1/M_s)$ was found to be 1.6 x
were obtained. Chart 4A shows the results due to treatment with UV or AAAF alone. From the shape of the curves we see, as expected, that the repair of AAAF damage was of the UV type (long-patch), since $A(1/M_a)$ versus dose is concave downward. From Chart 4A we calculated, with the model in Ref. 17, that the patches for UV- and AAAF-damaged DNA contain 40 BrdUrd residues or approximately 130 nucleotides with 40% S. E. in the case of UV and 80% S. E. in the case of AAAF. The number of repaired regions after 10 $\mu$M AAAF is approximately one-half of that after 10 J/sq m in agreement with the results obtained by unscheduled DNA synthesis measurements. Results of experiments carried out on cells exposed to a combined treatment (Chart 4B) are in general agreement with those in Chart 1. The amount of repair from a combined treatment is less than the sum of the individual treatments and, with the exception of the 10 J/sq m plus 10 $\mu$M data, are also as expected.

**Endonuclease-sensitive Sites.** The use of *M. luteus* endonuclease provides a sensitive way to detect pyrimidine dimers in DNA but not changes in DNA produced by AAAF. The DNA of human or hamster cells exposed to AAAF is not affected by the enzyme, nor does the AAAF-treated DNA inhibit UV endonuclease activity (1) (unpublished data). Chart 5 shows sedimentation data from cells treated with UV alone and incubated for 12 hr after irradiation. The dose used was 3.6 J/sq m. We calculated the number of sites removed from the values of $M_w$ given on Chart 5 and in its legend in the following way. At zero time, the number of endonuclease sites per dalton is given by $2(1/9.3 - 1/34.3) \times 10^{-8} = 15.8 \times 10^{-8}$. After 12 hr of incubation the number of sites per dalton equals $2(1/11.2 - 1/36.0) \times 10^{-8} = 12.2 \times 10^{-8}$. Hence, the number of sites removed per dalton during a 12-hr incubation is $(15.8 - 12.2) \times 10^{-8} = 3.6 \times 10^{-8}$. Finally, we did a number of experiments to determine the number of UV endonuclease-sensitive sites removed in 9 hr after a combined treatment with both agents as compared to UV alone (Chart 6). In order to get high sensitivity in these experiments, we mixed UV-irradiated cells (labeled with $[^{14}C]$thymidine) that were not incubated with treated incubated cells (labeled with $[^{3}H]$thymidine). The difference between the number of sites between $[^{14}C]$DNA and $[^{3}H]$DNA is the number of sites removed during the 9-hr incubation. The number of sites removed saturates at a dose of 5 J/sq m, and treatment with AAAF inhibits the removal of sites introduced by 3.6 J/sq m. Ten $\mu$M of AAAF causes ~55% inhibition of site removal.

**DISCUSSION**

V-79 cells do less excision repair of UV-irradiated DNA than do human fibroblasts as indicated by the difficulty of measuring excision directly (8) and the lower amounts of unscheduled DNA synthesis, BrdUrd photolysis, and removal of endonuclease sites compared to normal human cells (Ref. 1; present data). Unscheduled DNA synthesis and repair replication in V-79 were shown to saturate a UV dose of 20 J/sq m (4, 5). In that respect, they resemble normal human fibroblasts (1, 7, 27). No previous detailed studies were reported on the ability of these cells to perform exci-
Table 1  
Comparison between levels of unscheduled DNA synthesis in human fibroblasts and V-79 cells due to UV or AAF or a combined treatment

<table>
<thead>
<tr>
<th>Treatment (dose or concentration)</th>
<th>Human fibroblasts(^a)</th>
<th>Hamster cells(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV(10 J/sq m)</td>
<td>26</td>
<td>14</td>
</tr>
<tr>
<td>AAF(10 µM)</td>
<td>25</td>
<td>8.4</td>
</tr>
<tr>
<td>Combined(10 J/sq m + 10 µM)</td>
<td>47</td>
<td>5.3</td>
</tr>
</tbody>
</table>

\(^a\) Unscheduled DNA synthesis expressed as average number of grains per nucleus.

\(^b\) Cells left in Dulbecco's medium containing [\(^3\)H]thymidine, 2 µCi/ml, and 2 mM hydroxyurea for 3 hr before radioautographs were prepared (1).

\(^c\) Cells left in Dulbecco's medium containing [\(^3\)H]thymidine, 5 µCi/ml, and 4 mM hydroxyurea for 1 hr before radioautographs were prepared.

At high AAF concentrations it appeared that each agent and human cells after AAF or UV or a combined treatment the relative amounts of unscheduled DNA synthesis in hamster and human cells after AAF or UV or a combined treatment differed (Table 1). In a combined treatment, unscheduled DNA synthesis in V-79 cells was not additive and at high AAF concentrations it appeared that each agent inhibited the repair of the other. In normal human fibroblasts the combined treatment gave an additive effect (1), and we concluded that the repair of one did not affect repair of the other. With the use of the BrdUrd photolysis assay we found that the extent of repair of AAF damage was less than that of UV damage, even though AAF appeared to be repaired by a long-patch mechanism similar to UV repair. The photolysis assay also showed that repair due to both agents was much less than additive. To verify these results we used a UV endonuclease to measure the removal of pyrimidine dimers in cells given a combined treatment. Such an assay is much more sensitive than chromatographic determination of dimers and permits dimer determination as a result of UV alone or in the presence of AAF. We found that the number of nuclease-sensitive sites removed (2.5/10⁶ in 9 hr) was much less than in human fibroblasts (14/10⁶ in 6 hr in humans), and, moreover, that AAF treatment led to an inhibition of site removal in V-79 cells. In normal human fibroblasts, AAF did not affect removal of sites (1).

Excision repair measured radioautographically saturated around 20 J/sq m of UV, whereas dimer removal as determined by the endonuclease sites assay saturated around 2.5 J/sq m. We assume that the difference results from the fact that unscheduled DNA synthesis measured during the 1st hr after treatment gave the initial rate, whereas dimer removal measured 9 hr after treatment gave the extent of excision repair.

The decreased DNA repair observed when UV and AAF were applied cannot be due to cytotoxic effects leading to cell killing and detachment because we did not observe any detachments at the highest doses used, and we did not detect any decrease in radioactivity 12 hr after treatment with both agents in the BrdUrd photolysis assay.

Although chemicals such as AAF and 4-nitroquinoline 1-oxide mimic many of the biological effects of UV in human and in bacterials cells (6, 13, 15, 17, 24) and are like UV in that they are repaired by seemingly similar mechanisms, there is good recent evidence that AAF damage and UV damage are removed from DNA by different enzymatic steps (1, 2). Although there may be many steps in common in the 2 repair pathways, it seems as if the initial endonuclease step is different in vivo. In bacterial cells infected with T4 phage, the v gene of the phage codes for a protein that incises UV-irradiated phage DNA but not DNA treated with 4-nitroquinoline 1-oxide (11), and the presence of the v gene results in higher survival of UV-irradiated phage, but it does not lead to repair of 4-nitroquinoline 1-oxide-treated phage (13).

The picture for V-79 cells is complicated. Not only is the repair after a combined treatment of UV and AAF less than additive, but each agent seems to inhibit repair of the other. We speculate that there are different endonucleases that work at the 1st step in excision repair and that major or minor photoproducts inhibit the "AAAF endonuclease," and the major or minor AAF damage inhibits the "UV endonuclease" in V-79 cells. Such hypothetical, inhibitory products might explain the low level of excision repair of UV damage in hamster cells. In any case it is clear that not only are V-79 cells less proficient than normal human ones in repairing UV damage or AAF damage but that the relationships between enzymes in the 2 pathways are different from those in human cells.

REFERENCES

DNA Repair in V-79 Cells Treated with Combinations of Ultraviolet Radiation and \( N \)-Acetoxy-2-acetylaminofluorene

Farid E. Ahmed and R. B. Setlow


Updated version

Access the most recent version of this article at:

http://cancerres.aacrjournals.org/content/37/9/3414

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, use this link

http://cancerres.aacrjournals.org/content/37/9/3414.

Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.