Defective Removal of DNA Cross-Links in a Repair-deficient Mutant of Chinese Hamster Cells

Raymond E. Meyn, Susan F. Jenkins, and Larry H. Thompson

ABSTRACT

To further understand the relationships between DNA damage, DNA repair, and cellular end points such as survival and mutation, the repair capacity of a DNA repair-deficient mutant (strain UV-20) of Chinese hamster ovary cells was characterized in response to DNA cross-linking agents. This mutant, previously shown to be hypersensitive to killing by both ultraviolet light and the cross-linking agent mitomycin C, was also found to be extremely sensitive to cis-diamminedichloroplatinum, another DNA cross-linking agent. The efficiency of DNA cross-link removal after treatment with mitomycin C or cis-diamminedichloroplatinum was measured using the technique of alkaline elution and compared in wild-type Chinese hamster ovary cells and strain UV-20. Wild-type cells removed 80% or 95% of the cross-links within 24 hr after treatment with cis-diamminedichloroplatinum or mitomycin C, respectively. In contrast, UV-20 cells, which were equally as susceptible to cross-link damage as were wild-type cells, removed only a small proportion of the cross-links made by either agent. These results emphasize the importance of DNA repair processes in modulating the cytotoxic effects of chemicals that produce DNA cross-link damage and suggest that cross-link repair in Chinese hamster ovary cells is controlled by a pathway that also repairs damage from ultraviolet radiation.

INTRODUCTION

Relationships between the DNA lesions induced, the repair of those lesions at the molecular level, and the resulting genetic damage have been intensively studied in both microbial and mammalian systems exposed to UV radiation (9). Progress has also been made towards delineating cellular responses to DNA damage arising from chemical agents (16), although the details of repair of DNA adducts are poorly understood in somatic cells. Advances in understanding the role of DNA repair processes in the simpler systems have generally depended on the availability of repair-deficient mutants. Although numerous such mutants exist for bacteria (9) and simpler eukaryotes (3), investigations of mammalian cells have relied mainly on human cells derived from patients with genetic disease syndromes such as XP (2) and FA (8). The XP mutations, which have been classified into 7 genetic complementation classes (1,11), were found to be extremely sensitive to cis-diamminedichloroplatinum, another DNA cross-linking agent, the efficiency of DNA cross-link removal after treatment with mitomycin C or cis-diamminedichloroplatinum was measured using the technique of alkaline elution and compared in wild-type Chinese hamster ovary cells and strain UV-20. Wild-type cells removed 80% or 95% of the cross-links within 24 hr after treatment with cis-diamminedichloroplatinum or mitomycin C, respectively. In contrast, UV-20 cells, which were equally as susceptible to cross-link damage as were wild-type cells, removed only a small proportion of the cross-links made by either agent. These results emphasize the importance of DNA repair processes in modulating the cytotoxic effects of chemicals that produce DNA cross-link damage and suggest that cross-link repair in Chinese hamster ovary cells is controlled by a pathway that also repairs damage from ultraviolet radiation.

MATERIALS AND METHODS

Cell Growth and Labeling with Radioisotopes. The cell strains used in this study, designated A8B for the wild-type and UV-20 for the mutant, were grown as monolayer cultures in McCoy's Medium 5A supplemented with 15% fetal bovine serum. All incubations were carried out at 37°C in a humidified atmosphere of 95% air-5% CO2. Under these conditions, the cultures had doubling times of approximately 12 hr. Cells in exponential growth were used in all experiments. DNA was labeled by incubation with [2-14C]thymidine (0.01 µCi/ml; 50 Ci/mmol; Schwarz/Mann, Orangeburg, N. Y.) for 22 hr. This labeling period was followed by a 6-hr incubation in medium without label in order to chase the label into high-molecular-weight DNA.

Drug Treatment. cis-DDP was obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. A stock solution of drug was made by dissolving 10 mg of cis-DDP in 20 ml of 0.85% NaCl solution, and the appropriate dilutions were made in complete medium. MMC (Bristol Laboratories, Syracuse, N. Y.) stock solutions were made in distilled water and diluted into complete medium. Cells were exposed to MMC in the dark. All drug treatments were for 1 hr, and the treatments were terminated by rinsing the cell monolayers 3 times with 0.14 M NaCl-5 mM KCl-3 mM glucose-4 mM NaHCO3.

Alkaline Elution Analysis. The alkaline elution analysis used in this investigation was essentially identical to that developed by Kohn et al. (12–14). Briefly, cells were removed from the treatment dishes by trypsinization, and approximately 1 x 106 [14C]thymidine-labeled cells were diluted into 20 ml of cold phosphate-buffered saline (0.15 M NaCl-0.014 M KH2PO4-0.086 M K2HPO4) and gently impinged onto polyvinyl chloride filters (25 mm diameter; 2-µm pore size; Millipore Corp., Bedford, Mass.). The cells were then rinsed twice with 10 ml of cold dimers as well as on the lesions from many chemicals that produce bulky, helix-distorting adducts in the DNA (2). As a consequence of repair deficiency, XP cells are abnormally sensitive to the cytotoxic and mutagenic effects of such agents (15, 20, 24). FA cells, on the other hand, are characteristically hypersensitive to the cytotoxic effects of DNA cross-linking agents such as MMC and nitrogen mustard (8). As yet, there is no general agreement on the nature of the defect in FA (4, 8).

In an attempt to further understand the relationships between cross-link formation in DNA, the repair of these lesions, and cytotoxicity, we investigated the effects of MMC and cis-DDP in wild-type and repair-deficient CHO cells. The mutant strain UV-20 used in this study was isolated on the basis of hypersensitivity to killing by UV radiation and was shown to be deficient in repair replication in response to UV damage (23). UV-20 showed a remarkable degree of hypersensitivity to killing by MMC (80-fold) and belongs to the genetic complementation class that includes the majority of UV-sensitive mutants obtained in CHO cells (22). Here, we demonstrate that the extreme sensitivity of UV-20 to MMC extends to another cross-linking agent, cis-DDP; and we also show, using the technique of alkaline elution, that the mutant strain is defective in removing the cross-links formed by either of these agents.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: XP, xeroderma pigmentosum; FA, Fanconi's anemia; MMC, mitomycin C; cis-DDP, cis-diamminedichloroplatinum; CHO, Chinese hamster ovary.

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phosphate-buffered saline and treated with 5 ml of lysis solution (0.2% Sarkosyl-0.04 M EDTA-2 M NaCl, pH 10.0). The filters were then rinsed once with 5 ml of 0.02 M EDTA (pH 10.3). In this case, as with the lysis step, the solution was allowed to pass through the filter by gravity. The DNA on the filters was then eluted in the dark with 0.1 M tetrapropylammonium hydroxide-0.02 M EDTA (acid form) (pH 12.1) at a constant flow rate of 0.04 ml/min. Ten 4-ml fractions were collected. The radioactivity in these fractions and that remaining on the filter at the end of the elution were assayed by the liquid scintillation counting procedures of Kohn et al. (14).

In order to use the alkaline elution technique for the detection of drug-induced DNA cross-links, it was necessary to introduce single-strand breaks into the DNA with ionizing radiation. Cross-linking was then observed as a reduction in the elution of the DNA from cells that had been treated with the drug plus radiation compared to that from control cells that had received radiation alone (12). For this purpose, the cells were placed on ice and irradiated with 600 rads of X-ray (250 kVP) at a dose rate of 383 rad/min just prior to analysis by alkaline elution. After irradiation, the cells were kept on ice until lysis in order to prevent repair of the single-strand breaks. A relative cross-link factor was calculated from alkaline elution data by dividing the log of the fraction of DNA retained in the radiation control at the sixth fraction (9 hr) by the log of the fraction of DNA retained in the drug plus radiation sample at the same fraction (18). The values for relative cross-link factors obtained in this manner did not depend highly on which fraction was used.

In some experiments, the cell lysates were subjected to proteolytic digestion prior to elution (5). Proteinase K (0.25 mg/ml; Beckman Instruments, Inc., Palo Alto, Calif.) was added to the lysis solution described above in these cases, and this solution was allowed to remain in contact with the cells on the filter for 30 min at room temperature. The lysate was then rinsed with 0.02 M EDTA, and the DNA was eluted as described above. This procedure reduces the retention of protein on the filter to approximately 1% of total cell protein (12) and has been shown to effectively reverse the DNA-protein cross-linking produced by a number of agents including UV (5) and trans-diaminedichloroplatinum (25).

RESULTS

The cytotoxic effects of cis-DDP and MMC on wild-type (AA8) and repair-deficient (UV-20) CHO cells are presented in Chart 1 as dose-response curves for colony formation. Because of the very large differences in sensitivity between the 2 cell lines, the results are plotted in log-log form rather than in the usual semilog form. As can be seen, the repair-deficient cell line was extremely sensitive to both drugs compared to the wild type. The ratio of doses required to reduce cell survival to 37% for AA8 versus UV-20 cells was about 100 for MMC and about 70 for cis-DDP.

The sensitive technique of alkaline elution originally described by Kohn et al. (13, 14) was used to determine whether the differences in sensitivity to cross-linking agents displayed by the AA8 and UV-20 cell lines in Chart 1 were due to differences in the amount of DNA damage produced or to subsequent repair of that damage. Results from typical experiments in which UV-20 and AA8 cells were treated with various doses of cis-DDP or MMC for 1 hr and then analyzed immediately by alkaline elution are presented in Chart 2. In these graphs, the fraction of DNA retained on the filter is plotted against the time of elution (or fraction number). As described in "Materials and Methods," all of the cell samples received a dose of ionizing radiation just prior to lysis; cross-linking was then reflected by the degree of reduction in the rate of elution of the broken DNA from drug-treated cells as compared with DNA from cells that received only the radiation. The degree of this retardation of the rate of elution increased as the drug dose increased (Chart 2). Relative cross-link factors (see "Materials and Methods") calculated from several alkaline elution profiles similar to those in Chart 2 are plotted in Chart 3 as a function of drug dose. The degree of cross-linking produced in the AA8 and UV-20 cell lines by either cis-DDP or MMC was similar within experimental error.

While the initial level of cross-linking produced in the DNA of AA8 and UV-20 cells was nearly the same, the kinetics of cross-link removal was quite different. The kinetics of formation and disappearance of cross-linking in the DNA of AA8 and UV-20 cells at various times following treatment with cis-DDP or MMC is shown in Chart 4. For cells treated with cis-DDP, the cross-linking increased over the first 4 hr after drug removal as the second arm of the cross-links formed through a slow reaction (26). Thereafter, the cross-linking decreased; but, the decrease was much more pronounced in AA8 cells than in UV-20 cells. AA8 removed nearly 80% of the maximum amount of cross-links produced in their DNA by 24 hr after drug treatment. UV-20 cells, on the other hand, only removed about 24% of the maximum amount of cross-links during the same period of time.

A similar result was seen for cells treated with MMC (Chart 4). In this case however, all the cross-links were produced during the 60 min of drug treatment. Again, AA8 was much more capable of removing cross-links from DNA than was UV-20. AA8 cells removed about 95% of the damage by 24 hr after the drug was removed, whereas UV-20 cells removed only...
about 35%.

cis-DDP is known to produce 2 different types of cross-links involving DNA, i.e., interstrand cross-links and DNA-protein cross-links, which can be distinguished by alkaline elution (25). These lesions can be differentiated in the alkaline elution procedure by the use of a proteolytic digestion step after the regular lysis step (5). Cross-links removed by the procedure are presumed to be of the DNA-protein type, and cross-links remaining after this step are thought to be the DNA-interstrand type. Results demonstrating the proportion of total cross-linking produced by cis-DDP or by MMC that is proteolytic digestion resistant are presented in Table 1. Several points can be made concerning these data. (a) By comparison of the values given in Table 1 to those for total cross-linking displayed in Chart 3, it is evident that, for cells treated with either cis-DDP or MMC, the proteolytic digestion step removed some of the cross-linking. About one-half and one-fourth of the cross-links were removed by proteinase from the DNA of cells treated with cis-DDP and MMC, respectively. (b) As was the case for total cross-linking (Chart 4), AA8 cells were proficient in removing proteinase-resistant cross-links produced by either drug from their DNA, whereas UV-20 cells have reduced ability to remove cis-DDP-induced proteinase-resistant cross-links and apparently no capability for removing MMC-induced proteinase-resistant cross-links.

**DISCUSSION**

In the absence of additional information, the extreme cytotoxicity of cis-DDP and MMC in the UV-20 cell line as compared with the response of AA8 cells (Chart 1) could be explained by a variety of reasons, and the experiments described in this study were undertaken in order to understand the possible mechanism for these effects in more detail. The rationale for the experiments involved a comparison of the amount of DNA damage produced and the subsequent repair of that damage in the 2 cell lines after exposure to the 2 agents, with the assumption that DNA cross-links produced by these drugs are the primary lesions responsible for their cytotoxicity. Measurements using the technique of alkaline elution of DNA to determine the amount of cross-linking present as a function of drug concentration (Chart 3) showed that the amount of cross-linking was essentially the same in the UV-20 and AA8 cell lines immediately following the 1-hr drug treatment. These results imply that the large differences in cell killing observed when UV-20 and AA8 cells are exposed to these agents are not due to excessive cross-linking in the DNA of UV-20 cells, which might result from increased uptake of drug or a decreased rate of drug inactivation. Instead, the repair kinetics experiments (Chart 4) suggest that UV-20 has a reduced capacity for removing cross-links from its DNA during the 24 hr following drug treatment. In contrast, the parental AA8 cells appear to be quite efficient in this process, removing the majority of the lesions within the first 12 hr. Because the particular mutant used, UV-20, has been characterized previously as deficient in excision repair of UV damage in DNA, these findings suggest that cis-DDP and MMC exert their lethality via damage to DNA and that a functional DNA repair process can greatly modify the cytotoxic effects of these compounds.

An interesting aspect of cross-link formation by cis-DDP is that cross-links continue to form in the DNA for at least 4 hr after the drug is removed from the cells (Chart 4). This phe-

### Table 1

<table>
<thead>
<tr>
<th>Drug treatment (µg/ml)</th>
<th>Cell line</th>
<th>Repair time (hr)</th>
<th>Relative cross-linking</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-DDP (5)</td>
<td>AA8</td>
<td>4</td>
<td>1.70 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>AA8</td>
<td>24</td>
<td>0.97 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>UV-20</td>
<td>4</td>
<td>2.25 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>UV-20</td>
<td>24</td>
<td>1.60 ± 0.04</td>
</tr>
<tr>
<td>MMC (2.5)</td>
<td>AA8</td>
<td>0</td>
<td>2.75 ± 0.60</td>
</tr>
<tr>
<td></td>
<td>AA8</td>
<td>24</td>
<td>1.15 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>UV-20</td>
<td>0</td>
<td>2.60 ± 0.60</td>
</tr>
<tr>
<td></td>
<td>UV-20</td>
<td>24</td>
<td>2.90 ± 0.10</td>
</tr>
</tbody>
</table>

* Mean ± S.E. of 3 independent experiments.
Defective Repair of DNA Cross-Links in Mutant Cells

nomenon has been described by Zwelling et al. (26) and is presumably due to the slow conversion of cis-DDP monoadducts, produced in the DNA during the drug treatment, into interstrand DNA cross-links. Based on this mechanism, the difference in the level of maximum cross-links produced in the AA8 and UV-20 cell lines at 4 hr posttreatment with cis-DDP (Chart 4) can be explained by assuming that the repair process is active in AA8 cells starting at time zero and can remove the monoadducts as well as cross-links. Thus, 2 competing reactions might be going on during the first 4 hr, one involving formation of cross-links from monoadducts and one involving repair of monoadducts or cross-links or both. The result would be that more cross-links would be present in the repair-deficient cell line by the end of the first 4 hr compared to the normal cells. The small additional amount of cross-linking observed in UV-20 cells at time zero in Chart 4 (and Chart 3) may be explained on the same basis, by assuming that some repair occurs during the treatment interval.

The ability of mammalian cells to remove cis-DDP- and MMC-induced cross-links from their DNA has been demonstrated by other investigators. Zwelling et al. (26) using alkaline elution have shown that mouse L1210 cells remove the DNA cross-links produced by cis-DDP within 18 to 24 hr after treatment (26). Fraval and Roberts (7), while not examining cross-linking directly, have shown a correlation between the ability of Chinese hamster V-79 cells in different growth phases (exponential versus stationary) to remove bound platinum from their DNA and their relative sensitivity to the drug. Fraval et al. (6) have also reported that XP fibroblasts are 4-fold more sensitive to killing by cis-DDP than are normal human cells. However, the ability of the XP cells to remove bound platinum from DNA was not examined.

Previous studies of the repairability of MMC-induced DNA cross-links in FA fibroblasts have yielded conflicting conclusions. Fujiwara et al. (8) found that several strains of FA fibroblasts were hypersensitive to killing by MMC and deficient for repair of MMC cross-links compared to normal human cells. They also observed that XP cells behaved normally with respect to cell killing and repair of cross-link damage after MMC treatment. However, Fornace et al. (4), using a different technique and a different strain of FA cells that was only moderately hypersensitive to killing by MMC, demonstrated a normal removal of MMC-induced cross-links.

It is not yet known whether genetic heterogeneity underlies the FA syndrome, but the sensitivity of different FA strains to cross-linking agents does vary considerably (8, 19). In any event, the UV-20 CHO strain represents a phenotype that is distinct from both XP and FA cells since it is markedly hypersensitive to killing by both UV and MMC (22) and it is deficient in repairing lesions produced by each of these agents. In this regard, UV-20 is phenotypically analogous to the uvr mutants of Escherichia coli (9).

MMC and cis-DDP, because of the quite different way in which they interact with DNA to produce interstrand cross-links (16), were chosen to test whether the repair system in CHO cells would favor removal of the lesions produced by one agent over the other. The results (Chart 4) suggest that AA8 cells can remove the cross-links produced by either agent with apparently equivalent efficiency. UV-20 cells do not repair either class of lesions efficiently. However, since cis-DDP and MMC make a variety of different lesions in DNA, including interstrand DNA cross-links, DNA-protein cross-links, monoadducts and, in the case of cis-DDP, intrastrand cross-links (17), the question is raised as to which of these lesions contribute to the cytotoxicity of the drug under consideration. The findings presented here do not presume to fully answer this question, but they do demonstrate a strong positive correlation between the ability of a cell to remove interstrand cross-links from DNA via the excision repair pathway and resistance to killing by the drug treatment. This correlation can be made only on a qualitative basis because the level of residual unrepaired cross-linking is too low to be measured in wild-type cells after 24 hr of repair time and therefore cannot be compared on a quantitative basis with repair-deficient cells. Our results (Table 1) also showed that the wild-type CHO cells were able to remove essentially all measurable proteinase-resistant cross-links by 24 hr after treatment with either cis-DDP or MMC. In the case of MMC, UV-20 had no detectable repair, which may account for its extreme sensitivity to killing (Chart 1). These results also suggest that proteinase-resistant cross-link repair is highly efficient in wild-type CHO cells.

The UV-20 strain has been shown by Thompson et al. (23) to be deficient in excision repair of UV-induced photoproducts and also chemical adducts of 7-bromomethylbenz(a)anthracene (21). Thus, repair of the lesions produced in DNA by cis-DDP or MMC may involve at least one step in common with UV excision repair pathway. Isolation of a revertant clone from UV-20 in which resistance to both UV and MMC is restored suggests that the mutation in UV-20 is of the point-mutation type rather than a deletion, supporting the idea that a mutation in a single gene produces sensitivity to both UV and cross-linking agents. In this respect, UV-20 cells may be similar to certain strains of XP. For example, Kaye et al. (10) studied normal human and XP cells treated with 8-methoxypsoralen, a drug which intercalates into DNA and produces interstrand cross-links and monoadducts upon exposure of the cells to near-UV, and angelicin, which makes only monoadducts. The amount of repair replication induced in normal cells was the same for the 2 drugs, and no repair replication was induced by either compound in XP cells. These results suggested that the repair replication in the normal cells in response to 8-methoxypsoralen is primarily a response to the monoadducts and that this repair pathway shares at least one step with repair of pyrimidine dimers.

It seems reasonable to us, based on the observations of Kaye et al. (10), to assume that cells which cannot remove interstrand cross-links because of a defective excision repair system are also unable to remove monoadducts or intrastrand cross-links. Thus, regardless of which of these lesions is responsible for the drug cytotoxicity, the observations reported here demonstrate the important role of DNA repair mechanisms in modulating the cytotoxic effects of the anticancer drugs cis-DDP and MMC. Furthermore, since the UV excision repair system in both human and hamster cells appears to act on a variety of DNA lesions, DNA repair-deficient mutants such as the one used in this study become powerful new tools for furthering our understanding of the relationships between DNA damage and repair and the cellular end points of cytotoxicity, mutagenesis, and carcinogenesis.

* L. H. Thompson, unpublished observations.
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