Comutagenic Effects of 3-Aminobenzamide in Chinese Hamster Ovary Cells


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ABSTRACT

Inhibition of poly (ADP-ribose) synthesis by agents such as 3-aminobenzamide (3-AB) potentiates the cytotoxic, carcinogenic, and clastogenic effects of certain DNA-damaging agents. Experiments were carried out in Chinese hamster ovary cells to compare chromosome aberration production and cytotoxicity with the induction of somatic mutations at the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and sodium-potassium ATPase loci after treatment with 3-AB in combination with certain monofunctional alkylating agents. On its own, 1 to 10 mw concentrations of 3-AB were not mutagenic, reduced plating efficiencies only slightly, and produced a small elevation in the frequency of chromatid aberrations. In combination with ethyl methanesulfonate (EMS), 3-AB increased cytotoxicity and the frequency of alkyltion-induced chromatin aberrations. 3-AB also increased the frequency of EMS and N-methyl-N'-nitro-N-nitrosoguanidine-induced 6-thioguanine-resistant cells (a marker for the HGPRT- phenotype). It had no effect on the frequency of EMS-induced ouabain-resistant cells (a marker for ATPase mutations). All the effects were dose dependent. Larger absolute increases were found with 10 mw 3-AB as compared with 1 mw 3-AB and with 2 mw EMS as compared to 1 mw EMS. The 3-AB-mediated increases in 6-thioguaine-resistant cells, which are often deletion mutations, and the lack of any increase in the frequency of ouabain-resistant cells, which can only arise through point mutation induction, along with the increases in chromosome aberration frequency, suggests that 3-AB increases the frequency of deletion mutations by increasing the frequency and duration of DNA strand breaks.

INTRODUCTION

3-AB3, a potent inhibitor of poly(ADP-ribose) polymerase (24, 30), has a number of pleiotropic effects on cells. 3-AB will delay cell cycle progression (27), increase sister chromatid exchange frequency (13, 24, 27), inhibit de novo nucleotide synthesis (19) and cell differentiation (1, 11, 14), and potentiate the cytotoxic (8, 9) and clastogenic (21) effects of certain DNA damaging agents. In addition, 3-AB has been shown to be a cocarcinogen, potentiating the carcinogenic effects of alkylating agents both in vitro (18), and in vivo (26, 31). The mechanism for the potentiation of mutagen-induced cytotoxicity, clastogenicity, and carcinogenicity is believed to be a 3-AB-mediated delay in the rate of ligation of DNA strand breaks. DNA ligase II activity is stimulated by ADP-ribosylation and, in the presence of 3-AB, the rate of ligation is slower (7). This delay in strand break rejoining has been shown with ionizing radiation (32) and with certain alkylating agents (9, 20).

3-AB will increase the frequency of both chromosome deletions and exchanges in cells exposed to ionizing radiation or alkylating agents (21). Increases in the frequency of chromosome aberrations, such as rearrangements and deletions, might be expected to result in increased mutation frequency, because mutations at some loci, such as the HGPRT locus, for example, are often deletion mutations, even when induced by chemical carcinogens (6). In a preliminary study (28), we found 3-AB to be a comutagen in CHO cells, increasing the frequency of 6-TG-resistant cells (a marker for HGPRT activity) when combined with certain monofunctional alkylating agents. In the present study, we have continued to examine the question of mutation induction in CHO cells exposed to combinations of alkylating agents and 3-AB to determine whether the response is related to the 3-AB-mediated increase in chromosomal deletions. To do this, we have compared the mutation response to chromosome aberration production and cytotoxicity and, more particularly, we have looked at the induction of OUA-resistant cells after treatment with combinations of 3-AB and a monofunctional alkylating agent to determine whether 3-AB can affect the frequency of point mutations. OUA-resistant mutations which occur at the sodium-potassium ATPase locus cannot be deletion mutations (2).

MATERIALS AND METHODS

Culture Conditions, Survival, and Mutation Assays. CHO cells (AA8) were maintained under exponential growth conditions in McCoy's 5A medium supplemented with 10% (v/v) fetal calf serum, penicillin (50 units/ml), streptomycin (50 µg/ml), and 2 mM L-glutamine. Because of the expected high toxicity associated with combinations of alkylating agents and 3-AB, large numbers of cells were treated. Ten million cells were plated in 850-sq cm roller bottles containing 100 ml of medium; after 24 h, when the cultures were 50% confluent, the medium was replaced with fresh medium, and the cells were treated with the mutagen in the presence or absence of 3-AB. The mutagens used were EMS (1 or 2 mw) and MNNG (3.4 µM). Chemical treatments were nearly simultaneous, although mutagens were added to the cultures first. After 24 h of treatment, the cells were washed twice in saline, and the cells were removed by trypsin treatment. For survival measurements, six 100-mm Petri dishes were inoculated with 250 or 1000 cells per plate in 10 ml complete medium. These were held at 37 °C in a humidified 5% CO2 atmosphere for 10 to 12 days at which time the colonies were fixed in 3:1 methanol-acetic acid and stained in 5% Giemsa; then, colonies containing more than 50 cells were counted.

The induced mutation frequency was determined at 2 loci, the HGPRT and the sodium-potassium ATPase loci. To measure the mutation fre-
frequency at the HGPRT locus, more than 10^7 cells were transferred into 850-sq cm roller bottles with 50 ml of complete medium and maintained under exponential growth conditions for 8 days, the previously determined expression time (29). A minimum of 10^7 cells were transferred every 2 to 3 days. Although OUA resistance has been reported to have a short expression time, we found no difference in induced mutation frequency at this locus in CHO cells over a 2- to 8-day period, and we therefore used an 8-day expression time for measurement of OUA resistance. After 8 days in culture, plating efficiencies were again determined as described above. In addition, the frequency of cells resistant to either 6-TG (6-TG<sup>6</sup>) or OUA (OUA<sup>6</sup>) were determined. Resistance to 6-TG or to OUA were used as markers for mutations at the HGPRT (25) and ATPase (2) locus, respectively. The frequency of 6-TG<sup>6</sup> cells was measured by plating 50,000 to 100,000 cells into 10 ml of medium containing dialyzed serum and antibiotics and supplemented with 10 μM 6-TG. At least 5 plates/point were established in each experiment. To determine the frequency of OUA<sup>6</sup> cells, 10<sup>6</sup> cells were plated into each of 5 plates/point in complete medium supplemented with 3 mM OUA. These were held at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere for 10 to 12 days, at which time the colonies were fixed, stained, and counted. Mutation frequencies per viable cell (F) were calculated according to the formula:

\[ F = \frac{N}{(N_0 \times PE)} \]

where \( N \) is the total number of colonies observed, \( N_0 \) is the number of cells plated in selective medium, and PE is the surviving fraction of cells grown in nonselective medium and determined at the same time.

Chromosome Aberration Determinations. Exponentially growing cells were exposed to EMS and 3-AB for 15 h. Colcemid (2 × 10<sup>-7</sup> m final concentration) was added for the last 2 h. Mitotic cells were collected by shaking, treated with 0.075 M KCI for 15 min to spread the chromosomes, and fixed with 3:1 methanol:acetic acid. Chromosomes were stained for 2 min in a 7% Giemsa solution, and 100 cells per experiment were analyzed for each treatment. The metaphase chromosomes were examined for chromatid breaks and chromatid exchanges (triradials, quadriradials, and exchanges involving more than 3 chromosome arms).

RESULTS

Survival. As has been reported with other alkylating agents and inhibitors of poly(ADP-ribose)polymerase (8, 9), when CHO cells were exposed for 24 h to 1 to 2 mM EMS in the presence of 1 to 10 mM 3-AB, plating efficiency decreased dramatically as compared to cells treated with the mutagen alone (Chart 1). By itself, 3-AB reduced plating efficiency only marginally. The 3-AB-related increases in cytotoxicity after exposure to EMS were directly related to both the dose of mutagen and the dose of 3-AB. With larger doses of EMS, larger absolute increases in cytotoxicity were seen after 3-AB treatment. Similarly, at equivalent EMS exposures, survival decreased as a function of 3-AB dose. Exposure of CHO cells to 3.4 μM MNNG in the presence of 10 mM 3-AB decreased plating efficiency from 73 to 13%.

Chromosome Aberration Frequency. A 15-h exposure to 3-AB increased the base-line aberration frequency by primarily increasing the frequency of chromatid-type breaks (Table 1). Cells were harvested after 15 h to ensure that most cells were in their first division after treatment. Chromosome aberration frequency tends to decline with excessive cell divisions, as cells with aberrations die and chromosome fragments are lost. The response was dose-dependent but small. EMS is a potent clastogen and, when EMS was combined with 3-AB, the frequency of aberrations increased. The increases were dependent on the dose of 3-AB. One mM 3-AB had a small effect on the aberration frequency, increasing the frequency of EMS-induced breaks and exchanges only slightly. Ten mM 3-AB, while having little additional effect on break frequency, greatly enhanced the frequency of exchanges 2- to 5-fold over what was found with EMS alone. Surprisingly, addition of 10 mM 3-AB to cells treated with 1 or 2 mM EMS resulted in similar frequencies of aberrant cells, i.e., the frequency of aberrations was similar in cells treated with 1 mM EMS and 10 mM 3-AB to cells treated with 2 mM EMS and 10 mM 3-AB.

Mutation Induction. Combining 3-AB treatment with exposures to either EMS or MNNG resulted in an increase in the frequency of 6-TG<sup>6</sup> cells (Table 2). 3-AB by itself had no effect on the base-line frequency. As with the other phenomena studied, the effect was dependent on both the dose of mutagen and the dose of 3-AB. The absolute increases after treatment with 1 mM EMS and 1 mM 3-AB were small but significant, while 10 mM 3-AB doubled the frequency of 6-TG<sup>6</sup> cells when combined with 1 or 2 mM EMS. Similarly, 10 mM 3-AB doubled the frequency of MNNG-induced 6-TG<sup>6</sup> cells. Some of the 6-TG<sup>6</sup> clones were isolated and maintained to determine if the phenotype was stable.
COMUTAGENIC EFFECTS OF 3-AB

Table 1

<table>
<thead>
<tr>
<th>Mutagen treatment</th>
<th>Aberrant cells</th>
<th>Breaks</th>
<th>Exchanges</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1 mM EMS</td>
<td>10</td>
<td>10</td>
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<tr>
<td>2 mM EMS</td>
<td>20</td>
<td>16</td>
<td>13</td>
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<td>4 mM EMS</td>
<td>39</td>
<td>46</td>
<td>25</td>
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<tr>
<th>Mutagen treatment</th>
<th>Aberrant cells</th>
<th>Breaks</th>
<th>Exchanges</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM EMS + 10 mM 3-AB</td>
<td>15</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>2 mM EMS + 10 mM 3-AB</td>
<td>49</td>
<td>42</td>
<td>40</td>
</tr>
<tr>
<td>3.4 μM MNNG + 10 mM 3-AB</td>
<td>52</td>
<td>31</td>
<td>46</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Mutagen treatment</th>
<th>6-TG&lt;sup&gt;a&lt;/sup&gt;</th>
<th>OUA&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.7 ± 1.5&lt;sup&gt;c&lt;/sup&gt; (9&lt;sup&gt;d&lt;/sup&gt;)</td>
<td>59 ± 3.2 (3)</td>
</tr>
<tr>
<td>1 mM 3-AB</td>
<td>2.8 ± 0.4 (8&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>4.5 ± 2.8 (4)</td>
</tr>
<tr>
<td>10 mM 3-AB</td>
<td>3.2 ± 0.3 (10&lt;sup&gt;f&lt;/sup&gt;)</td>
<td>3.1 ± 1.7 (3)</td>
</tr>
<tr>
<td>1 mM EMS</td>
<td>49.0 ± 2.2 (12)</td>
<td>74.1 ± 3.7 (3)</td>
</tr>
<tr>
<td>1 mM EMS + 1 mM 3-AB</td>
<td>64.4 ± 4.6 (4&lt;sup&gt;g&lt;/sup&gt;)</td>
<td>82.7 ± 3.6 (3)</td>
</tr>
<tr>
<td>1 mM EMS + 10 mM 3-AB</td>
<td>79.3 ± 5.9 (12)</td>
<td>68.7 ± 9.9 (3)</td>
</tr>
<tr>
<td>2 mM EMS</td>
<td>123.0 ± 22.2 (2)</td>
<td></td>
</tr>
<tr>
<td>2 mM EMS + 10 mM 3-AB</td>
<td>232.6 ± 4.2 (2&lt;sup&gt;g&lt;/sup&gt;)</td>
<td></td>
</tr>
<tr>
<td>3.4 μM MNNG</td>
<td>139.4 ± 10.6 (2)</td>
<td></td>
</tr>
<tr>
<td>3.4 μM MNNG + 10 mM 3-AB</td>
<td>295.1 ± 18.1 (2)</td>
<td></td>
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</table>

<sup>a</sup> Frequency of 6-TG<sup>b</sup> cells per 10<sup>6</sup> clonable cells.
<sup>b</sup> Frequency of OUA<sup>a</sup> cells per 10<sup>6</sup> clonable cells.
<sup>c</sup> Mean ± SE.
<sup>d</sup> Numbers in parentheses, number of experimental determinations.
<sup>e</sup> Statistically different from the mutagen-alone treatment (P < 0.05).
<sup>f</sup> Statistically different from the mutagen-alone treatment (P < 0.01).
<sup>g</sup> Statistically different from the mutagen-alone treatment (P < 0.001).

DISCUSSION

As in an earlier report of ours (28), we have found an inhibitor of poly(ADP-ribose) synthesis, 3-AB, to be a comutagen, potentiating the mutagenic as well as cytotoxic and clastogenic effects of alkylating agents. On its own, 3-AB reduced plating efficiency by less than 10%, increased the base-line frequency of chromatid breaks only slightly, and was not mutagenic. These effects have been noted previously (22, 27). When 3-AB was combined with the alkylating agents MNNG or EMS, the frequency of 6-TG<sup>b</sup> mutants was doubled, plating efficiency was sharply reduced and, in the case of EMS, the frequency of chromatid aberrations increased greatly.

Recently, Bhattacharya and Bhattacharjee (30) reported that benzamide, another poly(ADP-ribose) synthesis inhibitor, reduced the frequency of MNNG-induced azaguanine-resistant V79 cells. Differences exist among various cell types in their response to 3-AB or benzamide (4, 5, 9), and modifications in procedures such as the use of 8-azaguanine instead of 6-TG (22) could produce different results. Therefore, the difference between our report and theirs may be due to the difference in cells used or the different procedures used. Alternatively, the small number of cells surviving the combination of benzamide and MNNG exposure could have biased their result. Bhattacharya and Bhattacharjee only exposed 10<sup>6</sup> cells for mutation analysis. The high toxicity of the combined treatment of CHO cells with 3-AB and alkylating agents coupled with the relatively low frequency of OUA<sup>a</sup> mutants prevented us from examining the effect of this treatment on OUA<sup>a</sup> after exposures to 2 mM EMS or 3.4 μM MNNG.

Increased damage by another agent after treatment with a mutagen is often attributed to an inhibition of repair processes. The incision step of excision repair is not believed to be affected by 3-AB because, after exposure to various alkylating agents, the removal of alkylated bases is not inhibited by either 3-AB (5) or 5-methyl nicotinamide (10), another potent inhibitor of poly(ADP-ribose) polymerase. Furthermore, CHO cells are normally deficient in the ability to excise О<sup>6</sup>alkylguanine (12), a major premutagenic lesion induced by alkylating agents in mammalian cells (16, 17), and it also appears to be totally lacking in any premutational repair (15).

Five to 10 mM concentrations of 3-AB, however, appear to inhibit the ligation step in DNA repair (7, 8). Specifically, 3-AB decreases DNA ligase activity and thereby delays the rejoining of DNA strand breaks. Such a delay in strand break rejoining would not only increase the number of DNA strand breaks found at any given time, as Morgan and Cleaver have shown (20), but would also allow more time for the formation of abnormal rearrangements between broken strands and thus increase the frequency of chromatid aberrations, as seen in Table 1. An increase in the frequency of DNA strand breaks would also account for the 3-AB-mediated increase in cytotoxicity and the increase in 6-TG<sup>b</sup> mutants, because mutations at the HGPRT locus, as measured by resistance to 6-TG, are often deletions (6) and are induced by clastogenic agents such as ionizing radiation.

In contrast to mutations at the HGPRT locus, mutations at the ATPase locus as measured by OUA resistance can only be point mutations and not the result of deletions (2). DNA strand breaks, such as those produced by ionizing radiation, do not affect the frequency of OUA<sup>a</sup> mutants. The fact that 3-AB had no significant effect on the EMS-induced frequency of OUA<sup>a</sup> cells indicates that the comutagenic effect noted here is not a consequence of an increase in point mutations but is probably due to an increase in the frequency of DNA strand breaks.

In conclusion, 3-AB will potentiate the mutagenic effects of nonmonofunctional alkylating agents, as well as their clastogenic, cytotoxic, and carcinogenic effects. While the magnitude of the 3-AB-mediated potentiation varies with each end point, the most likely target for all appears to be DNA strand break ligation.

REFERENCES

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