Inhibitory Effect of Reducing Agents on N-Acetoxy- and N-Hydroxy-2-acetylanilinofluorene-induced Mutagenesis

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ABSTRACT

The effect of cysteine (α-amino-β-mercapto-propionic acid) on the mutagenic activities of the proximate carcinogen, N-hydroxy-2-acetylaminofluorene, and the ultimate carcinogen, N-acetoxy-2-acetylanilinofluorene, was examined by estimating the frequency of his revertants of Salmonella typhimurium. Nontoxic concentrations of cysteine significantly reduced the formation of revertants when it was applied concurrently with the two carcinogens. Cysteine showed no detectable effect on mutagenesis when added to bacteria before or after exposure to carcinogens. The magnitude of inhibition of mutagenesis depended on the dose of cysteine and the concentration of the carcinogens. Cysteine at equimolar concentrations inhibited to a larger degree the mutagenesis induced by N-hydroxy-2-acetylaminofluorene than it inhibited that elicited by N-acetoxy-2-acetylanilinofluorene. The inhibitory action of cysteamine and glutathione was comparable to that of cysteine. The results appear to be consistent with the assumption that cysteine traps electrophiles prior to their action on DNA.

INTRODUCTION

The use of trapping agents with carcinogen-inhibiting properties is a promising area of study that has not been fully explored (6, 7, 10, 17, 19, 22-25). The lack of information on trapping agents is partly due to the difficulty of obtaining results when animals are used to examine the multitude of interactions that arise when several agents are administered. A battery of short-term tests were recently designed and successfully applied in the screening of compounds for their mutagenic-carcinogenic capacity (e.g., Refs. 12, 13, 20, and 21). These tests should also be applicable to the detection of agents that inhibit mutagenesis and oncogenesis (11). We have previously shown the usefulness of DNA fragmentation and DNA repair synthesis of cultured human fibroblasts in quantitating the action of ascorbate during nitrosation of methylguanine (9, 21) and its inhibiting effect on dimethylnitrosamine (10). In this paper we describe the application of the Ames mutagenesis test (1-3) to uncover and quantitate the inhibitory effect of cysteine (α-amino-β-mercapto-propionic acid) on the mutagenic actions of the potent carcinogen N-acetoxy-AAF² and the precarcinogen N-hydroxy-AAF⁴.

MATERIALS AND METHODS

A histidine-requiring strain of Salmonella typhimurium, TA98 (obtained from Dr. B. Ames, University of California at Berkeley, Berkeley, Calif.), was used as an indicator organism for mutagenic activity. Frozen master stock was inoculated into nutrient broth and grown overnight on a rotary wheel (31 rpm) in an incubator (37°). Aliquots (0.1 ml) of this stationary-phase growth were reinoculated into fresh nutrient medium (5 ml). These latter cultures were grown for 4 hr (ca. 70 to 100 × 10⁶ cells/ml) prior to use. Cultures were vortexed, 1-ml aliquots were placed into centrifuge tubes, and the bacteria were pelleted with a clinical centrifuge (5000 rpm for 5 min). Pellets were resuspended in treatment media (total volume of 1 ml) and placed in the incubator (37°) for 20 min. At the end of this time, the bacteria were pelleted by centrifugation, washed with PBS, and resuspended in PBS (1 ml total volume). Aliquots from each sample were assayed for viability and for the amount of reversion to histidine independence.

Assays for reversion frequency were performed by suspending 0.1 ml of each sample in 2 ml top agar (1, 3) and by overlaying this mixture on minimal agar plates (1, 3). The bacteria samples were diluted with 0.85% NaCl solution and plated on nutrient agar plates (1, 3) for survival studies. All agar plates were incubated at 37° for 48 hr prior to scoring. Mutagenic activity was calculated in terms of the number of revertants per 10⁷ surviving bacteria cells.

Stock solutions of N-acetoxy-AAF and N-hydroxy-AAF were prepared in dimethyl sulfoxide/ethanol (1/9) followed by dilution with PBS. Cysteine, glutathione, and cysteamine were dissolved directly into PBS. All solutions were adjusted to pH 7.4 before use. In treatments that involved the use of reducing agents in conjunction with carcinogens, the 2 solutions were mixed together just before the bacteria were resuspended in the mixture.

RESULTS

Inhibition of N-Acetoxy-AAF-induced Mutagenesis with Cysteine. The mutagenic action of N-acetoxy-AAF on S. typhimurium was markedly inhibited if cysteine was concurrently applied (Chart 1B). The degree of the inhibition of the mutagenic action of N-acetoxy-AAF was dependent on the concentration of cysteine used. Furthermore, the amount of inhibition varied with the concentration of N-acetoxy-AAF.

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² Supported by grant from the National Cancer Institute of Canada.
³ Research Associate of the National Cancer Institute of Canada. To whom requests for reprints should be addressed.
⁴ The abbreviations used are: N-acetoxy-AAF, N-acetoxy-2-acetylanilino-
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The mutagenic action of 50 and 100 μM N-acetoxy-AAF (Table 1).

Pretreatment and Posttreatment with Cysteine. The effect of cysteine pretreatment and posttreatment on N-acetoxy-AAF-induced mutagenesis was examined (Table 2). There was no inhibition of mutagenic action in samples exposed to a 20-min pretreatment with cysteine before exposure to N-acetoxy-AAF. Similarly, posttreatment with cysteine failed to prevent the mutagenic action of N-acetoxy-AAF. Reduction in N-acetoxy-AAF-induced mutagenesis occurred only if the carcinogen and the reducing agent were added concurrently to the bacteria.

Table 1

<table>
<thead>
<tr>
<th>Reducing agent</th>
<th>N-Acetoxy-AAF (100 μM)</th>
<th>N-Acetoxy-AAF (50 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>309.8 ± 33.2</td>
<td>187.5 ± 8.1</td>
</tr>
<tr>
<td>Cysteine (10 mM)</td>
<td>50.6 ± 3.2</td>
<td>24.7 ± 3.6</td>
</tr>
<tr>
<td>Glutathione (10 mM)</td>
<td>45.8 ± 3.3</td>
<td>38.0 ± 2.6</td>
</tr>
<tr>
<td>Cysteamine (10 mM)</td>
<td>35.3 ± 5.2</td>
<td>35.6 ± 2.5</td>
</tr>
<tr>
<td>Cysteine (5 mM)</td>
<td>57.2 ± 5.1</td>
<td>45.8 ± 5.9</td>
</tr>
<tr>
<td>Glutathione (5 mM)</td>
<td>47.7 ± 7.4</td>
<td>39.1 ± 0.2</td>
</tr>
<tr>
<td>Cysteamine (5 mM)</td>
<td>59.0 ± 4.0</td>
<td>37.3 ± 7.4</td>
</tr>
<tr>
<td>Cysteine (2.5 mM)</td>
<td>122.0 ± 5.0</td>
<td>55.0 ± 0.6</td>
</tr>
<tr>
<td>Glutathione (2.5 mM)</td>
<td>71.4 ± 5.3</td>
<td>69.6 ± 5.5</td>
</tr>
<tr>
<td>Cysteamine (2.5 mM)</td>
<td>77.4 ± 9.6</td>
<td>91.1 ± 4.7</td>
</tr>
</tbody>
</table>

* Value for control samples (PBS only) was 4.2 ± 2.0.

Table 2

The inhibitory effect of several reducing agents on the mutagenic action of N-acetoxy-AAF.

<table>
<thead>
<tr>
<th>Reducing agent</th>
<th>Mutagenic activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>12.0 ± 2.0</td>
</tr>
<tr>
<td>Cysteine (10 mM)</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>Glutathione (10 mM)</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>Cysteamine (10 mM)</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>Cysteine (5 mM)</td>
<td>6.0 ± 1.0</td>
</tr>
<tr>
<td>Glutathione (5 mM)</td>
<td>5.0 ± 0.5</td>
</tr>
<tr>
<td>Cysteamine (5 mM)</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>Cysteine (2.5 mM)</td>
<td>12.0 ± 2.0</td>
</tr>
<tr>
<td>Glutathione (2.5 mM)</td>
<td>6.0 ± 1.0</td>
</tr>
<tr>
<td>Cysteamine (2.5 mM)</td>
<td>5.0 ± 0.5</td>
</tr>
</tbody>
</table>

a Value for control samples (PBS only) was 4.2 ± 2.0.
b Mean ± S.D.; n = 3.
c Number in parentheses, percentage of activity.
**Table 2**

Effect of timing on inhibitory effect of cysteine on N-acetoxy-AAF-induced mutagenesis

Samples of *S. typhimurium* (40 x 10^7 cells) were exposed to one of the following treatments: (a) pretreatment with cysteine prior to exposure to N-acetoxy-AAF; (b) posttreatment with cysteine following N-acetoxy-AAF treatment; (c) treatment with N-acetoxy-AAF alone, pre- and posttreatments with PBS; and (d) treatment with N-acetoxy-AAF in conjunction with cysteine, pre- and posttreatments with PBS. Pretreatment, treatment, and posttreatment periods were each 20 min long. Samples were centrifuged down and resuspended in PBS between pretreatment, treatment, and posttreatment periods. Cysteine concentration used was 10 mM.

<table>
<thead>
<tr>
<th>Mutagenic activity (revertants/10^7 survivors) when cysteine applied&lt;sup&gt;a&lt;/sup&gt;</th>
<th>N-Acetoxy-AAF concentration (µM)</th>
<th>No cysteine</th>
<th>Prior to carcinogen</th>
<th>Concurrent to carcinogen</th>
<th>After carcinogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50</td>
<td>33.1 ± 3.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.7 ± 2.6</td>
<td>8.7 ± 2.6</td>
<td>30.3 ± 1.6</td>
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<tr>
<td></td>
<td>40</td>
<td>19.6 ± 2.7</td>
<td>17.4 ± 1.2</td>
<td>6.3 ± 0.8</td>
<td>18.6 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>11.1 ± 1.8</td>
<td>12.3 ± 1.3</td>
<td>4.6 ± 1.5</td>
<td>12.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>10.4 ± 3.3</td>
<td>9.2 ± 0.8</td>
<td>5.1 ± 1.4</td>
<td>10.5 ± 1.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± S.D.; n = 4.

**DISCUSSION**

Nontoxic concentrations of cysteine significantly reduced the formation of revertant cells in *S. typhimurium* cultures that were exposed to the potent frame-shift mutagens (12, 13) and carcinogens (14–16) N-hydroxy-AAF and N-acetoxy-AAF. The degree of inhibition of this mutagenic activity depended on the dose of cysteine, the concentration of the mutagen, and the time of cysteine application. Cysteine showed no detectable effect if it was added to the bacteria prior to or after their exposure to the 2 mutagens.

The simplest explanation of these results is that cysteine “traps” the mutagen and/or the reactive species of the mutagen. Cysteine could prevent the formation of the free nitrooxide radical by reacting directly with the mutagen. Interaction of cysteine with the nitrenium ion stage of the electrophilic intermediate (4, 5, 18) would also prevent the interaction of this molecule with DNA. One could propose other mechanisms for the cysteine inhibition of mutagenesis. Cysteine could enhance the repair of the conformational distortion of DNA caused by N-acetoxy-AAF treatment (8). However, there is no evidence in support of this action. An inhibition of DNA repair is suggested by the reduction by cysteine of the unscheduled incorporation of [3H]thymidine in UV-irradiated human fibroblast cultures (P. Lam, personal communication). As an alternative theory, cysteine inhibition of mutagenesis could result from an inhibition of DNA replication by cysteine combined with a cell cycle-specific action of N-acetoxy-AAF. A blockage of DNA replication by cysteine could have a preventive effect on mutagenesis if N-acetoxy-AAF would react only with replicating DNA. However, the capacity of N-acetoxy-AAF to produce DNA damage in nondividing mammalian cells is well documented (8, 21).

There are several possible explanations for the differential inhibition by cysteine of equivalent mutagenic activities (revertants/10^7 survivors) of N-hydroxy-AAF and N-acetoxy-AAF. N-Hydroxy-AAF (which must be esterified to the N-acetoxy form to be active) may be more easily trapped by cysteine because of its extended presence in the system (4, 5, 14–16, 18, 26). Cysteine may prevent the additional activation step(s). However, bacteria may be unable to esterify the N-hydroxy-AAF in the absence of mammalian liver extract and thus may rely on other activation systems, such as an H2O2-peroxidase-type mechanism (5). Thus, N-hydroxy-AAF could be activated to esters other than N-acetoxy-AAF. If so, the varying reactivities of these esters would, in turn, affect the amount of cysteine required to trap them.

The application of the Ames mutagenesis assay (13) as well as the DNA fragmentation and DNA repair assays (20, 21) may prove invaluable in the search for compounds that can abolish the mutagenic actions of carcinogens. Moreover, these short-term assays would provide a technique of measuring the comparative efficiency of such “trapping” compounds. A correlative study involving a larger series of carcinogens and electron scavengers may allow us to predict whether these short-term assays will indicate the inhibitory action of trapping agents in vivo carcinogenesis.

**ACKNOWLEDGMENTS**

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**REFERENCES**

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