ABSTRACT

Hycanthone, the most potent mutagen in a series of nine thiaxanthenones, is a potent inducer of nuclear immunoreactivity to antinucleoside antibodies in HeLa cells. This response indicates exposure of single-stranded DNA regions. All classes of mutagens thus far tested share this property with hycanthone. Immunoreactivity to antinucleoside antibodies was also induced by brief exposure to hycanthone, 3 μg/ml, in human fibroblasts from three normal subjects and in fibroblasts from seven patients with DNA repair deficiencies.

Unlike those of many other mutagens, the metabolic effects and immunoreactivity induction of hycanthone were readily reversible. No evidence for covalent attachment of [3H]hycanthone to HeLa macromolecules could be found. Induction of DNA repair synthesis could not be detected by autoradiography after exposure of cells to hycanthone. Exposure of single-stranded DNA regions appears to be an important feature of the mechanism of action of hycanthone as a mutagen.

Both hycanthone and lucanthone intercalate with DNA, but hycanthone was much less active than was lucanthone in reducing the rapid sedimentation of cell lysate DNA in alkaline sucrose gradients. Similarities and differences, therefore, have been found in the way the potent and the weak mutagen affect DNA of HeLa cells. This may provide clues to understanding the mechanism of mutagenesis by thiaxanthenones and other mutagens.

INTRODUCTION

Immunoreactivity to antinucleoside antibodies is detectable almost exclusively during S phase in cells from cultures or animal and human tumor biopsies (5, 22, 25, 26). This reaction is readily detectable with fixed cells with the use of either immunoperoxidase or immunofluorescent techniques. The molecular basis of the S phase-related nuclear immunoreactivity is believed to be due to single-stranded DNA regions, which normally occur during replication. The specificity of antinucleoside antibodies for single-stranded nucleic acids was demonstrated by Erlanger and Beiser (16) and Klein et al. (22) and has been abundantly confirmed by many others (24).

In certain situations induction of nuclear immunoreactivity unrelated to semiconservative DNA synthesis can be detected. Agents that damage DNA, such as ionizing radiation (2, 5, 26) and chemical carcinogens (6), induce immunoreactivity in G1-phase cells. The immunoreactivity is probably due to single-strand breaks, which expose single-strand regions by partial unwinding of the helix at the site of the breaks. Local denaturation and excision repair processes presumably follow, but it is not yet known to what extent the exposed single-strand regions themselves or the action of DNA excision repair enzymes is responsible.

Cycloheximide and other protein synthesis inhibitors not known to be carcinogens also induce immunoreactivity to antinucleoside antibodies in G1 HeLa cells. These protein synthesis inhibitors induce immunoreactivity that is rapidly reversible upon removal of the inhibitor (6). The mechanism by which protein synthesis inhibition leads to nuclear immunoreactivity is not yet understood but is being studied in our laboratory.

Results of immunoreactivity induction tests made with certain chemical carcinogens were difficult to interpret because, in addition to reacting covalently with macromolecules and inducing DNA strand breaks, they are also potent inhibitors of protein synthesis (6).

To avoid interference from inhibition of protein synthesis, we chose to study a series of thiaxanthenones. Hycanthone, the potent frame-shift mutagen, is the most potent mutagen of this group (7, 8, 11, 19, 21). The mutagenicity of thiaxanthenones has been detected in eukaryotic as well as prokaryotic systems, especially with the widely used histidine revertant test as described by Hulbert et al. (21). Hycanthone has distinct potential as a carcinogen (9, 17, 18). Both lucanthone (Miracil D) and hycanthone intercalate with DNA. Both have antischistosome and antitumor properties, which were recently reviewed (20); they have been widely used in the clinical treatment of human schistosoma parasites; serum concentrations of 3 μg/ml are commonly achieved in patients (20).

Hycanthone (Chart 1) differs from lucanthone in that the 4-methyl group is replaced by an hydroxymethyl group. Structures of the thiaxanthenones studied (Table 1) may be found in Refs. 7, 11, and 20. Lucanthone is the direct metabolic precursor of hycanthone and is a much weaker frame-shift mutagen (19). Neither drug inhibits total cellular protein synthesis at 3 μg/ml in HeLa cells (4, 34). They both selectively inhibit rRNA but not mRNA synthesis at this concentration and do not cause loss of cell viability during 2 hr of exposure. Inhibition of rRNA synthesis and DNA synthesis by these drugs is rapidly and completely reversible. Parallel studies of lucanthone with hamster cell cultures gave similar results (15).

Tests with all thiaxanthenones described in this report were made at 3 μg/ml. We studied the influence of hycanthone and related thiaxanthenones on: (a) the induction of immunoreactivity to antinucleoside antibodies in HeLa cells; (b) the sedimentation properties of cell lysate DNA.

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2 Recipient of a Jane and Arnold Ginsburg Fellowship in Radiology. To whom requests for reprints should be addressed, at Department of Radiology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, N.Y. 10461.
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isolated from drug-treated HeLa cells (6, 14); (c) the incorporation of radioactive precursors into total cellular DNA, RNA, and protein in HeLa cells; (d) the ability of [3H]hycanthone to form covalent attachments to macromolecules in HeLa cells; (e) DNA repair synthesis induction (by hycanthone) measured by [3H]dThd autoradiography; and (f) the susceptibility of human fibroblasts to immunoreactivity induction by hycanthone to verify that this could be observed in cultures recently established from normal subjects and from those with DNA repair deficiencies.

MATERIALS AND METHODS

HeLa cells were grown in suspension, as described previously (2). Fluorescein-labeled rabbit antibodies against bovine albumin nucleoside conjugates were prepared by the methods described by Erlanger and Beiser (16). Details are given in our previous publications (2, 6, 26). Throughout these studies fluorescein-labeled antiguanosine at a 1:60 or 1:50 dilution was used. Similar results have been observed with antibodies specific for other nucleosides. The appearance of the specific nuclear immunoreactivity was indistinguishable from that demonstrated previously (2, 5, 26). Briefly, the cells were fixed in 100% acetone at -20° for 3 min after drying on glass slides. After staining, nuclear immunofluorescence (discrete intranuclear fluorescence) was scored with a Zeiss UV microscope. At least 200 cells were scored for each determination. Interference from nRNA was not encountered; pretreatment of fixed HeLa cells with pancreatic (2 ¡g/ml) and T-1 (2 units/ml) RNase at 20° for 30 min did not influence determination of immunoreactivity; results with human fibroblasts were similar. Similar pretreatment with S-1 nuclease resulted in complete loss of immunoreactivity.

Determination of the effects of agents on the rapidly sedimenting cell lysate DNA "complex" is based on the methods of Elkind (13) as adapted to HeLa cells (2, 3). For DNA sedimentation studies HeLa cell cultures growing randomly at a concentration of about 10^5 cells/ml were labeled overnight with [methyl-3H]thymidine (60 Ci/m mole) at 0.25 ¡Ci/ml. After resuspension in unlabeled Eagle’s growth medium (12), the cells were incubated for at least 1 hr before treatment with the drugs. The treated cells were washed twice with buffered 0.9% NaCl solution and lysed for 60 min at 20° on alkaline sucrose gradients. Centrifugation was then carried out at 6000 rpm in the Spinco SW-50.1 rotor (Beckman Instruments, Palo Alto, Calif.) for 17 hr at 0°. The radiolabel recovered from the gradients was at least 80% of the input to the gradient (at least 5000 cpm).

Incorporation of [3H]dThd, [3H]uridine, and [3H]-labeled amino acids into randomly growing HeLa cell macromolecules during exposure to thiaxanthenones was determined as described previously (4). The thiaxanthenones were dissolved in water, sterilized by filtration, and diluted just prior to use. With hycanthone and lucanthone, frozen solutions appear to be stable over long periods, and no difference in results was detected when freshly dissolved or frozen stock solutions were used. The concentrations of lucanthone and hycanthone were verified by examination of the absorbance at 432 nm (32). For hycanthone 1 mg/ml of the base yields an absorbance of 18.0. Methanesulfonate salts were used.

For autoradiography HeLa cells were labeled for up to 1 hr with 2 ¡Ci/ml, [3H]dThd, after which the radiolabel was removed and the cells were incubated for 15 min in growth medium containing 2 ¡m unlabeled thymidine. Autoradiographs were made using NTB 2 emulsion (Eastman Kodak Co., Rochester, N. Y.) and 48 to 72 hr of exposure. Only lightly labeled cells were scored, ignoring heavily labeled S-phase cells; only cells with 3 to 40 grains were scored as positive.

Human fibroblast lines obtained from the American Type Culture Collection or the Institute for Medical Research, Camden, N. J., were stored frozen in liquid nitrogen. These cells were cultured in Dulbecco’s modification of Eagle’s minimal essential medium containing 10% heated fetal bovine serum. For the studies of immunoreactivity induction, the cells were grown on glass slides for several days and then were exposed to hycanthone for 30 min before fixing.

Tritium-labeled hycanthone methanesulfonate was prepared by New England Nuclear, Boston, Mass. by catalytic exchange with tritium gas at room temperature. We purified the material by preparative thin-layer chromatography on silica gel plates developed with A/-hexane:chloroform:triethylamine (70:26:4). The specific activity was 6.29 mCi/mg.

RESULTS

Reversibility of Immunoreactivity Induction by Hycanthone. G1 HeLa cells were harvested by selective detachment and exposed to hycanthone, 3 ¡g/ml, in suspension. The cells were exposed to hycanthone for 30 or 40 min. Results of 2 experiments are shown in Chart 2. The high level of immunoreactivity induced in these cells was similar to results obtained in our previous studies (6). Hycanthone was removed by sedimenting the cells at low speed and resuspending them in normal growth medium. At the times shown the cells were again tested for immunoreactivity. Return to normal G1 levels of immunoreactivity (10 to 20% positive cells) was completed in approximately 1 hr, which is the time needed for the reversibility of the inhibitory effects of the drug on macromolecular synthesis (34).
### Table 1

<table>
<thead>
<tr>
<th>Thianthrene</th>
<th>Macromolecular synthesis: Fraction of control values</th>
<th>DNA complex</th>
<th>Immuno-reactivity</th>
<th>Mutagenicity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>DNA</td>
<td>RNA</td>
<td>Protein</td>
<td>DNA</td>
</tr>
<tr>
<td>Hycanthone</td>
<td>0.28</td>
<td>0.22</td>
<td>1.00</td>
<td>0.99</td>
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<tr>
<td>Lucanthone</td>
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<td>0.35</td>
<td>1.00</td>
<td>0.65</td>
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<tr>
<td>IA-4</td>
<td>0.10</td>
<td>0.11</td>
<td>0.72</td>
<td>1.08</td>
</tr>
<tr>
<td>IA-4 N-oxide</td>
<td>0.61</td>
<td>0.72</td>
<td>1.12</td>
<td>1.05</td>
</tr>
<tr>
<td>IA-3</td>
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<td>0.56</td>
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<td>NT</td>
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<tr>
<td>N-methyl lucanthone</td>
<td>0.88</td>
<td>0.76</td>
<td>1.19</td>
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<tr>
<td>N-methyl chlorolucanthone</td>
<td>0.40</td>
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<tr>
<td>N-methyl chlorolucanthone</td>
<td>0.97</td>
<td>0.94</td>
<td>1.12</td>
<td>0.94</td>
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</tbody>
</table>

* Incorporation of [³H]dThd, [³H]uridine, and [³H]-labeled amino acids in 30-min pulse-labeling experiments. Results of 2 to 3 separate experiments are shown (usually triplicate determination). Consult Ref. 4 for details.
* Relative moment of sedimentation: gradient fraction that divides the radiolabel into equal upper and lower portions, divided by the value for the control gradient.
* Percentage of positive cells above the G₀ cell background (about 10%). Cells were exposed to drugs for 30 min.
* Ames test using *S. typhimurium* strain TA-1538. Data taken from Hulbert et al. (21).
* Each compound was tested with 2 strains of *Salmonella*, TA-100 and TA-98, with and without rat liver microsomes, to activate the mutagens. Only the highest value obtained under these 4 conditions is shown. Without microsomes lucanthone was not active (i.e., >1 colony/nmole). This data was generously contributed by R. Batzinger and E. Bueding (personal communication).
* NT, not tested.

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**Hycanthone Mutagenesis**

**Table 1. Correlation of effects of thianthreneones, 3 µg/ml, in HeLa cells with mutagenicity in *S. typhimurium***

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**Hycanthone Does Not Attach Covalently to HeLa Cell Macromolecules.** HeLa cells in exponential growth (10⁸) were exposed to 15 mCi of purified [³H]hycanthone for 2 hr at 37°C. Following this cells were washed in buffered 0.9% NaCl solution, and their nuclei were prepared using the NP-40 detergent method (4). The DNA was purified by exhaustive extraction with phenol at 60°C followed by banding in CsCl density gradients. Cytoplasmic RNA was recovered by phenol extraction and examined by glycerol gradient centrifugation (4).

Nonhistone chromatin proteins which are tightly bound to DNA were prepared by the methods of Pederson and Bhorjee (29) from [³H]hycanthone-labeled cells. Other cells were similarly labeled, and total lipids were extracted by a Folch procedure with chloroform:methanol (2:1) and separated on silica gel plates (first dimension, chloroform:methanol:H₂O (70:25:4); second dimension, chloroform:methanol:NH₄OH (60:35:4)).

In these experiments we found no evidence for covalent attachment of [³H]hycanthone to DNA (<2 molecules/10⁷ bases), rRNA, tightly bound nonhistone chromatin proteins, or any of the major polar lipid fractions.

**Hycanthone Does Not Affect the Sedimentation of Cell Lysate DNA.** The cell lysate DNA complex is believed to contain duplex DNA (31) as well as single-stranded DNA (28). Normally, the DNA complex is about 10⁹ daltons (13). We previously reported that lucanthone was much more active than was hycanthone in dissociating the DNA complex (3). Because these results were unexpected and may provide a clue to differences in the mechanisms of action of the 2 drugs, we repeated and confirmed those experiments. Results are shown in Chart 3. The sedimentation studies were made at relatively low speed to avoid anomalous retardation of the complex (13).

Studies of single-stranded DNA, sedimented by standard alkaline sucrose gradient techniques at higher speed, failed to demonstrate DNA single-strand breaks in HeLa cells exposed to either lucanthone (3) or hycanthone (not shown).

**Correlation of Mutagenicity, Inhibition of Macromolecular Synthesis, and Effects on Macromolecules of a Series of Thianthreneones.** The data of Table 1 show that hycanthone was the most potent of the mutagens tested and that it induced a strong immunoreactivity response without inhibiting protein synthesis. Lucanthone and some other thianthreneones that did not inhibit protein synthesis did not induce immunoreactivity and were not strong mutagens. Certain thianthreneones did inhibit protein synthesis. As with cycloheximide, they may have induced immunoreactivity through the mechanism of protein synthesis inhi-
The values of immunoreactivity induction for pyrene and benzo(a)pyrene are probably overestimated in our experiments, since these drugs also caused an 18% inhibition of protein synthesis in HeLa cells. Protein synthesis inhibition may therefore have contributed to their relatively modest immunoreactivity induction in comparison with the active mutagens.

**Hycanthone Did Not Induce DNA Repair Synthesis as Determined by [³H]dThd Radioautography.** We found no increase in the background level of grains seen by radioautography of G, HeLa cells exposed to [³H]dThd during treatment with hycanthone, 3 µg/ml, for 1 hr or when labeled during the first hr after its removal. During hycanthone exposure 8.3% of the cells were lightly labeled; in the hr after removal 9.8% were labeled. This was not significantly different from control values of 6.6 and 12.0%, obtained with cultures treated equivalently but without the drugs. Three hundred to 800 cells were scored for each determination. Similar results were obtained with randomly growing HeLa cells. About 20% of the cells were positive after 1000 rads when X-rays were used to induce unscheduled (repair) synthesis.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Hycanthone-induced immunoreactivity to antiguanosine antibodies* in exponentially growing cultured cells from normal and DNA repair-deficient human subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>Subject</td>
</tr>
<tr>
<td>-----------</td>
<td>---------</td>
</tr>
<tr>
<td>CRL 1141</td>
<td>Normal</td>
</tr>
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<td>CRL 1147</td>
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<tr>
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<td>GM 648</td>
<td>Ataxia T</td>
</tr>
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<td>CRL 1346</td>
<td>Ataxia T</td>
</tr>
<tr>
<td>AG 1142</td>
<td>Retino B</td>
</tr>
<tr>
<td>CRL 1204</td>
<td>Xeroderma pigmentosum</td>
</tr>
</tbody>
</table>

*a* Fluorescein-labeled antiguanosine immunoglobulin, 1:50 dilution.

*CRL indicates American Type Culture Collection; GM and AG indicate Institute for Medical Research.

Average of 2 experiments.

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induction was not restricted to long-established cell lines, such as HeLa.

DISCUSSION

These studies with thiaxanthenones extend results of our previous studies with other classes of mutagens (6); the results suggest a relationship between induction in human cells of nuclear immunoreactivity to antinucleoside antibodies and the mutagenic potential of the agents. In this work we compared the immunoreactivity induction of thiaxanthenones in human cells with their mutagenicity in the widely used Salmonella typhimurium revertant test (1). In the latter test the ability of an agent to induce revertants has been strongly predictive of carcinogenicity in animal cell systems. Little comparative mutagenicity data are yet available for the thiaxanthenones in animal systems. Clive (11) compared mutagenicity at the TK locus in mouse lymphoblasts with certain thiaxanthenones; he found complex mutagenic responses in that system. At high concentration hycanthone was more mutagenic than was lucanthone.4

Exposure of single-stranded DNA regions by local denaturation of the duplex or production of single-strand breaks are at least 2 mechanisms by which immunoreactivity induction might be accomplished with agents like hycanthone. Lucanthone, a relatively weak mutagen (Table 1), also intercalates with DNA, and its effects resemble the biochemical and biological effects of hycanthone in very many ways (20). However, equivalent concentrations of lucanthone did not induce immunoreactivity to antinucleoside antibodies in HeLa cells. [Its presence did enhance the immunoreactivity response of HeLa cells exposed to ionizing radiation (3)].

DNA single-strand breaks could not be detected in HeLa cells exposed for 1 hr to lucanthone (3) or hycanthone. Therefore, it seems more probable that denaturation was responsible for the immunoreactivity induction that we observed in HeLa cells and fibroblasts exposed to hycanthone.

It is also possible that hycanthone and other classes of mutagens lead to exposure of single-stranded DNA regions by inhibiting the synthesis of minor classes of nonhistone chromatin proteins, which are normally required to maintain DNA in the native state in the cell.

DNA denaturation occurs as a consequence of covalent attachment of certain carcinogens to DNA (27) with subsequent excision and repair synthesis (30). It also occurs with base displacement (33). Results obtained using AAF, a less active mutagen than N-acetoxy-2-acetylaminofluorene, and benzo(a)pyrene, a precursor of the more potent activated benzo(a)pyrene derivatives, clearly point to progressive increase in the ability of the agents to induce immunoreactivity to antinucleoside antibodies with increasing mutagenicity. DNA denaturation, therefore, appears to be a common feature of mutagen action on DNA.

Results of studies on the sedimentation properties of the cell lysate DNA complex in cells exposed to thiaxanthenones did not provide a simple correlation with mutagenicity or immunoreactivity induction. Lucanthone and N-methyl-chlorolucanthone led to dissociation of the complex, but the strongest mutagen we tested, hycanthone, had very little influence on the complex. This unexpected result with hycanthone was noted in many different experiments.

We have no explanation for this but speculate that the ability of lucanthone to dissociate the complex might be accounted for by induction of a very few single-strand breaks, too few to be detected by centrifugation of single-stranded DNA but sufficient to be detected by the more sensitive method of studying sedimentation of the complex. Such single-strand breaks might disturb short duplex regions crucial in maintaining the integrity of the complex but too short to be detectable by the nuclear immunofluorescence test.

Since hycanthone had little effect on the complex, its readily detected immunoreactivity induction in HeLa cells suggests that it may have led to denaturation of duplex regions not involved in maintaining the integrity of the complex.

The immunoreactivity response to hycanthone was also observed in relatively early passages of freshly established human fibroblast cell lines. The similar response in repair-deficient cells suggests that repair processes may not be involved. This is in accord with the absence of repair synthesis. Hycanthone's weak cytotoxicity, easily reversed metabolic effects, and effects on the state of DNA, as well as its known mutagenic and carcinogenic effects, make it and other thiaxanthenones attractive candidates for future studies on mechanisms of mutagenesis. Our results emphasize the potential usefulness of antinucleoside immunoreactivity as the basis for a rapid test for potential mutagens.

A weak mutagen such as lucanthone seems to interact with DNA in a different manner from the stronger mutagen, hycanthone. Lucanthone might lead to strand separation which yields DNA strand breaks resulting in dissociation of the DNA complex but not antinucleoside immunoreactivity or mutagenesis. Hycanthone seems to interact differently, leading to strand separation (or possibly uncovering of naturally occurring single-stranded regions), antinucleoside immunoreactivity, and mutagenesis but not strand breaks and dissociation of the DNA complex. Carchman et al. (10) also have reported differences between lucanthone and hycanthone; lucanthone increased the relative viscosity of DNA to a much greater extent than did hycanthone.

Our results demonstrate 2 new differences between the way hycanthone and lucanthone affect cellular DNA. Pursuing these differences may eventually provide clues to the mechanism of thiaxanthanenone mutagenesis and could be relevant to the mechanism of action with other classes of mutagens.

ACKNOWLEDGMENTS

We are indebted to: Dr. A. E. Farah, Sterling Winthrop Research Institute, Rensselaer, N. Y., for supplying hycanthone and lucanthone; Dr. E. Etlinger, Parke-Davis and Co., Ann Arbor, Mich., for IA-3, IA-4, and IA-4 N-oxide; and

4 Note added in review: Recently, we observed morphological transformation of C. Heidelberger's CSH mouse embryo cell line 107/B clone B after a 3-hr exposure to hycanthone, 6 μg/ml. In 2 experiments the transformation rate with hycanthone was 6 x 10^{-2} and 38 x 10^{-2}; with lucanthone or untreated cells, the rate was less than 1 x 10^{-2} in both experiments.
REFERENCES


Early Steps in Mutagenesis by Hycanthone


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