Characterization of Hepatic DNA Damage Induced in Rats by the Pyrrolizidine Alkaloid Monocrotaline¹

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ABSTRACT

Hepatic DNA damage induced by the pyrrolizidine alkaloid monocrotaline was evaluated following i.p. administration to adult male Sprague-Dawley rats. Animals were treated with various doses ranging upward from 5 mg/kg, and hepatic nuclei were isolated 4 hr later. Hepatic nuclei were used as the DNA source in all experiments. DNA damage was characterized by the alkaline elution technique. A mixture of DNA-DNA interstrand cross-links and DNA-protein cross-links was induced. Following an injection of monocrotaline, 30 mg/kg i.p., DNA-DNA interstrand cross-linking reached a maximum within 12 hr or less and thereafter decreased over a protracted period of time. By 96 hr postadministration, the calculated cross-linking factor was no longer statistically different from zero. No evidence for the induction of DNA single-strand breaks was observed, although the presence of small numbers of DNA single-strand breaks could have been masked by the overwhelming predominance of DNA cross-links. These DNA cross-links may be related to the hepatocarcinogenic, hepatotoxic, and/or antimitotic effects of monocrotaline.

INTRODUCTION

PAs³ are naturally occurring toxicants found in multiple genera and species of 8 families of plants. As such, they are indigenous to large areas of the globe. In general, these compounds are hepatic and pulmonary toxicants, although the toxic signs may vary as a function of the species studied, route of administration, dose, and dosing schedule. PAs constitute a significant health hazard to both humans and animals. As with many toxicants, the alkaloids themselves are not toxic but rather must be bioactivated to the ultimate toxicants (for review, see Ref. 21). Throughout biotransformation studies have been hampered due to difficulties in producing adequately radiolabeled PAs. Metabolism proceeds through a variety of routes, but the conversion to toxic intermediates is generally thought to be mediated by the hepatic cytochrome P-450 mixed-function oxygenase system (17). This route results in the production of pyrrole derivatives. These pyrroles may undergo electron shifts, losing an ester group(s) to form alkylation species. Potentially, bifunctional alkylation species may be produced. Due in part to this alkylation potential, several PAs have been screened for antitumor activity, some with promising results. One PA, indicine A/-oxide, is currently undergoing clinical trials (23). For most other PAs, however, the toxicity of the compounds has limited their clinical usefulness (3).

In addition to the toxicities of these compounds, PAs are known carcinogens in animal models (9). When parent alkaloids such as monocrotaline have been administered, hepatic and pulmonary tumors predominate. When dehydrotretorine, a deesterified pyrrole derivative, was studied, rhabdomyosarcomas developed locally at the site of injection (26). Schoental (25) has suggested an association between ingestion of PAs and the high incidence of hepatocellular carcinoma among natives of the Bantu tribe of South Africa. She acknowledges, however, the probable coconsumption of aflatoxins as well, precluding establishment of a cause-effect relationship. PAs are capable of inducing the transformation of cells in culture, using growth in semi-solid agar as a marker of transformation (28). Williams and Mori (31) have shown these compounds to be genotoxic in the hepatocyte primary culture/DNA-repair test. In addition, many of these compounds are mutagenic in modified Ames assays, although monocrotaline is not (33). Because these and other in vitro studies indicate an interaction of PAs or their metabolites with DNA, we have chosen to investigate the type(s) of DNA damage induced in vivo by the PA monocrotaline (Chart 1). To accomplish this, we have used the technique of alkaline elution originally developed by Kohn et al. (15).

MATERIALS AND METHODS

Chemicals. Monocrotaline was isolated by Soxhlet extraction and recrystalization from the seeds of Crotonalstis spectabilis (1) or purchased from Trans-World Chemicals, Inc., Washington, DC. Purity was confirmed by thin-layer and high-pressure liquid chromatographic analyses. Sarkosyl NL-97 was purchased from ICN Pharmaceuticals, Inc., Plainview, NY. Sucrose and trichloroacetic acid were obtained from Fisher Scientific Co., Pittsburgh, PA. Tetraethyiammonium hydroxide (20%), EDTA, and diamino benzene acid dihydrochloride were purchased from Aldrich Chemical Co., Milwaukee, WI. Spermine tetrahydrochloride, spermidine trihydrochloride, Tris base, disodium EDTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N"-tetraacetic acid, glycerol, phenylmethysulfonyl fluoride, 2-mercaptoethanol, lauryl sulfate, and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO. Swinnex filter units and polyvinyl chloride filters (diameter, 47 mm; pore size, 2.0 μm) were purchased from Millipore Corp., Bedford, MA. Polycarbonate filters (diameter, 47 mm; pore size, 2.0 μm) were purchased from Nucleopore Corp., Pleasanton, CA. Proteinase K was obtained from Beckman Instruments, Inc., Fullerton, CA.

Treatment of the Animals. Male Sprague-Dawley rats (150 to 250 g) were obtained from breeding colonies maintained by the Division of Animal Resources at the University of Arizona. Animals were allowed food (Wayne Lab Blox) and water ad libitum. Monocrotaline was dissolved in deionized water with addition of sufficient HCl to adjust the pH to 5 to 6 prior to i.p. injection. Control animals received injections of deionized water alone. All injections were timed to allow termination of animals at 8 a.m. Hepatic nuclei were isolated by the method of Woll et al. (32) and served as the source of DNA in all experiments.

Detection of DNA Damage by Alkaline Elution. DNA damage was
RESULTS

Injections of monocrotaline i.p. did not appear to induce DNA single-strand breaks at 4 hr postadministration with doses of up to 90 mg/kg or at 8 hr after a dose of 120 mg/kg (Chart 2). Furthermore, no evidence of DNA single-strand breaks was apparent in rat hepatic DNA when nuclei were isolated at various times through 120 hr following a 30-mg/kg dose of monocrotaline (data not shown). DNA single-strand breaks would be indicated by an increase in the rate of elution of DNA from the filter and consequent increase in the slope of the elution profile in treatment groups compared to control groups.

For the detection of DNA-DNA interstrand cross-links, Kohn et al. (15) used 3 Gy of X-irradiation to induce a background of DNA single-strand breaks. In our experiments to detect DNA-DNA interstrand cross-links, 15 Gy were used for this purpose. The rationale for this higher amount of radiation is shown in Chart 3. The buffer in which the nuclei are resuspended after isolation contains 25% glycerol. This buffer allows nuclei to be stored at -80° for at least 7 days without altering the elution profile, thus allowing assays to be repeated or allowing detection of multiple types of DNA damage in the same animal (30). As shown in Chart 3, when mouse leukemia L-1210 cells, a cell line which has been used extensively in alkaline elution studies, were suspended in growth medium and X-irradiated with 8 Gy, a significant amount of DNA damage in the form of DNA single-strand breaks was induced. Induction of a large number of DNA single-strand breaks facilitated detection of any radioprotective effect produced by suspending cells in another buffer. When these cells were suspended in freezing buffer prior to X-irradiation, the DNA was refractory to the induction of this DNA damage. Addition of 25% glycerol to the growth medium produced an identical radioprotective effect. Fifteen Gy was selected...

\[ X(t) = \frac{(1 - R(c))^0.5}{1 - R(t)} - 1 \]  

where \( X(t) \) is the amount of DNA cross-linking induced by treatment \( t \), \( R(c) \) is the fraction of DNA retained on the filter in control groups, and \( R(t) \) is the fraction of DNA retained on the filter in treated groups. DNA-protein cross-linking was estimated by comparing cross-linking factors from replicate experiments carried out in the absence (which measures total cross-linking) and presence (which measures DNA-DNA interstrand cross-linking) of a proteinase K digestion step. Data in this case are expressed as the difference in cross-linking factors in those replicate experiments.

To address the possibility that any DNA single-strand breaks that might be induced were due to necrosis, liver sections were retained from all animals for histopathological examination after formalin fixation and hematoxylin-eosin staining.

Results were statistically analyzed by a randomized block analysis of variance, and means were compared by the Student-Newman-Keuls test (27). Differences were regarded as significant if \( P < 0.05 \).
this time point were not observed (data not shown). DNA cross-linking is indicated by an apparent decrease in the number of X-ray-induced DNA single-strand breaks and therefore a decreased rate of elution of treatment group DNA from the filter relative to the elution from the filter of DNA from control groups. The cross-linking was quantified by Equation A, given in "Materials and Methods."

Experiments to characterize the temporal aspects of DNA-DNA interstrand cross-link induction and removal were conducted using a dose of monocrotaline, 30 mg/kg i.p. (Chart 5). Results suggest that maximal DNA-DNA interstrand cross-linking had either occurred by 4 hr or occurred between 4 and 12 hr. Thereafter, DNA-DNA interstrand cross-links were removed over a protracted period of time. By 96 hr postadministration, the DNA-DNA interstrand cross-linking factor was no longer statistically significant.

Significant DNA-protein cross-linking was also induced by monocrotaline at 4 hr after alkaloid administration by doses as low as 15 mg/kg (Table 2). As described in "Materials and Methods," DNA-protein cross-linking was estimated by the difference in cross-linking factors calculated from replicate experiments, differing only in the omission or inclusion of a proteinase K digestion step.

No histopathological evidence of cytotoxicity was seen at

as a dose of X-irradiation that induced significant DNA fragmentation in hepatic nuclei despite being suspended in this freezing buffer, as shown by the elution profile of the control group in Chart 4.

Administration of monocrotaline resulted in DNA cross-linking. DNA-DNA interstrand cross-links were induced in a dose-dependent manner at 4 hr after i.p. administration of monocrotaline from doses as low as 5 mg/kg through doses of 30 mg/kg (Chart 4; Table 1). Doses of monocrotaline greater than 30 mg/kg were tested, but further dose-dependent increases in cross-linking at
DISCUSSION

It is generally thought that the process for initiation of cancer by chemicals involves an interaction of that compound or one or more of its metabolites with cellular DNA (7, 22). Here we report that the hepatocarcinogen monocrotaline induces DNA damage in the primary target tissue of a sensitive species following in vivo administration. These data complement a report that 2 PAs, senecionine and seneciphylline, covalently bind to hepatic macromolecules, including DNA, when administered to mice (5). Other studies on the induction of DNA damage by PAs have been conducted in vitro (31) and/or have used chemically synthesized "metabolites" (19, 24). Results of in vitro studies with dehydroretronecine, a deesterified pyrrolic analogue of monocrotaline, suggest that 2 alkylation reactions are occurring (11). In vitro evidence has also been published suggesting the ability of a pyrrolyl derivative of monocrotaline to induce interstrand cross-linking in DNA (29). Results reported here are consistent with these previous reports and are the first to demonstrate the induction of DNA cross-links in vivo by a PA.

DNA single-strand breaks may be induced by a variety of mechanisms. Included in these are direct scission, conversion of apurinic sites, hydrolysis of alkali-labile sites, enzymatic DNA repair activity, and degradation of DNA secondary to cytotoxicity/cellular necrosis (2, 13). Despite repeated efforts with various doses and time points, evidence for DNA single-strand breaks could not be detected. There is a possibility that small numbers of DNA single-strand breaks are induced by monocrotaline, but their existence is masked by the predominance of DNA cross-links. Robertson (24) has reported that dehydroretronecine, a deesterified pyrrolic analogue of monocrotaline, produces N-2 deoxyguanosine adducts racemic about the C-7 position of the locene nucleus when incubated in vitro with deoxyguanosine. The N-2 adducts described by Robertson were reactive with the nucleophile 4-(p-nitrobenzylpyridine), suggesting the possibility of their further reaction, perhaps with a different base, to form a cross-link. Mattocks and Bird (19) have reported recently that dehydroretronecine also alkylates a variety of other nucleophiles in vitro, including certain purine and pyrimidine derivatives. Thymidine and uridine, however, were apparently not alkylated. The sites of alkylation were not determined. Taken together, these results suggest that a derivative of monocrotaline can alkylate a variety of nucleophilic sites in vitro and that these alkylation products have the potential for further reaction with another nucleophile. In our system, monocrotaline induces significant DNA cross-linking, both DNA-DNA interstrand and DNA-protein, in rat hepatic nuclear DNA when administered i.p. 4 hr prior to termination of the animals. These in vivo results are consistent with the above-mentioned in vitro data and support the hypothesis that monocrotaline acts as a bifunctional alkylating agent.

DNA cross-linking may be important in the toxicity and/or carcinogenicity of monocrotaline. DNA cross-linking has been associated with the cytotoxicity of several antitumor agents; including the haloethylnitrosoureas (6) and platinum(II) complexes (34, 35). Furthermore, in the case of the platinum(II) complexes, cytotoxicity correlates much better with the induction of DNA-DNA interstrand cross-linking than with DNA-protein cross-linking. At comparable drug concentrations, cis-diamminedichloroplatinum(II), a potent antitumor agent, induced considerably more DNA-DNA interstrand cross-linking and cytotoxicity than did the trans isomer, which is essentially inactive as an antitumor agent. In contrast, at the drug concentrations used, DNA-protein cross-linking was similar with the 2 isomers. When drug concentrations were adjusted to induce a similar degree of DNA-DNA interstrand cross-linking, cytotoxicities of the 2 isomers were comparable. In line with these studies, Leopold et al. (16) have shown that the mutagenicity and tumorigenicity of the cis isomer are much greater than those of the trans isomer, suggestive of a relationship between DNA-DNA interstrand cross-linking and these effects. The tumorigenicity or cytotoxicity of monocrotaline may also be related to the induction of DNA-DNA interstrand cross-links. It must be noted, however, that cis-diamminedichloroplatinum(II) is also reported to induce intrastrand cross-links (8), while the trans-diamminedichloroplatinum(II) isomer does not. It is possible therefore that this intrastrand cross-linking is responsible for some or all of these effects. Such intrastrand cross-linking is not detectable by alkaline elution. It is not known if monocrotaline induces DNA-DNA intrasstrand cross-links.

The time course of development and clearance of cross-links within a given system may vary dramatically as a function of the cross-linking agent. Kohn (14) described the kinetics of cross-link induction and removal in mouse leukemia L-1210 cells by nitrogen mustard and L-PAM. DNA cross-linking in response to nitrogen mustard developed and peaked rapidly and was essentially removed within 24 hr posttreatment. In contrast, cross-linking with L-PAM appeared to be maximal at approximately 8 to 10 hr and, while the data were reported only through 32 hr, the decrease in the amount of DNA cross-linking was more protracted. Qualitatively, the kinetics of cross-link development and clearance following monocrotaline appears to resemble those of L-PAM more closely. The time-course studies for the development and removal of DNA-DNA interstrand cross-links.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Δ Cross-linking factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>0.299 ± 0.040*</td>
</tr>
<tr>
<td>15 mg/kg</td>
<td>0.690 ± 0.091*</td>
</tr>
<tr>
<td>30 mg/kg</td>
<td>1.396 ± 0.203*</td>
</tr>
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\* Significantly different from control (p < 0.05).
are somewhat difficult to interpret in the in vivo system we have studied. This system allows determination of the amount of cross-linking present at any given time. While the dose of monocrotaline used in these studies, 30 mg/kg, was relatively low and should be metabolized and excreted rapidly, an in vivo system such as this precludes the possibility of a true pulse exposure. Therefore, at certain time points, new cross-links may be forming, while others are being removed.

DNA-DNA interstrand cross-links would interfere with DNA replication. Single doses of pyrrolizidine alkaloids have been shown to produce hepatic megalocytosis and have potent, prolonged antimitotic effects (4, 10). Pyrrole derivatives and synthetic pyrrole analogues have been reported to produce similar effects (18, 20). Mattocks (20) tested several of these pyrrolic compounds, some of which were bifunctional alkylating agents and others of which were monofunctional alkylating agents, in a rat liver cell line. Antimitotic activities were seen to vary as a function of the chemical reactivity of the pyrrole and as a function of whether the particular pyrrole was a mono- or bifunctional alkylating agent. While some antimitic activity was seen with the highly reactive monofunctional pyroles, the bifunctional compounds of similar chemical reactivities produced a much greater antimitic effect.

In summary, these studies have shown that the PA monocrotaline produces both DNA-DNA interstrand cross-links and DNA-protein cross-links. In contrast, no evidence for the induction of DNA single-strand breaks was observed. Induction of DNA-DNA interstrand cross-links was dose and time dependent, and the removal of these lesions was protracted. Induction of DNA cross-links may be important in the antimitic, hepatoxic, and/or hepatocarcinogenic activity of monocrotaline.

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REFERENCES

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