Induction of Limited DNA Damage by the Antitumor Agent Cain’s Acridine

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ABSTRACT

The antitumor drug methanesulfonyl-m-anisidine, 4'-(9-acridinylamino) monohydrochloride (AMSA) (Cain’s acridine, NSC 141549), causes a limited, partially reversible decrease in size of the DNA of mouse L1210 leukemia cells as analyzed by centrifugation of cell lysates on alkaline sucrose gradients. Exposure of cells for 30 min to AMSA, 2.5 μg/ml, in tissue culture or to 150 μg/mouse in vivo results in a shift of long-term prelabeled DNA from >170S fractions to a broad band of about 30S. Higher doses or longer exposure times do not produce DNA much smaller than 30S. Labeled AMSA cosediments with heavy DNA in neutral sucrose gradients in which no degradation is observed, but AMSA is dissociated from both heavy and 30S DNA in alkaline sucrose gradients and appears only at the top of the gradient. On neutral gradients degradation to 30S DNA after AMSA exposure is detected only when cells are previously lysed in an alkaline medium but not when they are lysed in a neutral 70% formamide solution or when the neutral lysate is heated to 83° before centrifugation. Treatment of L1210 cells with other DNA intercalating agents (ethidium bromide, acridine orange, and phosphine orange) caused a similar degradation of the DNA on alkaline sucrose gradients. The results are interpreted to indicate that AMSA causes alkali-sensitive lesions of DNA at a limited number of sites; this effect correlates with the antitumor action of the drug and with other work showing long-term damage to chromosome structure.

INTRODUCTION

AMSA bearing i.p. L1210 cells (4% survived 50 days versus an average of 9.2 days for untreated controls). Other derivatives were more potent against s.c. or i.c. implanted L1210 cells.

We have shown that AMSA at lower doses inhibits the incorporation of labeled dThd into the DNA of L1210 cells when tested either in vivo or in tissue culture. For example, i.p. injection of 100 to 150 μg (5.0 to 7.5 mg/kg) of AMSA in an L1210 tumor-bearing mouse 1 hr before a 30-min period of [14C]dThd incorporation resulted in a 60 to 80% decrease in the amount of label taken up into the DNA of the tumor cells compared to controls without drug. Little effect on incorporation of labeled uridine or hypoxanthine into RNA was observed. Similar results were obtained with L1210 cells in primary cell culture; the presence of 2 to 4 μg of AMSA per ml for 1 hr before the addition of [14C]dThd for 30 min caused a decrease of 60 to 70% in the amount of label incorporated. In 48-hr cultures of L1210 cells, 0.05 μg/ml inhibited growth and 0.25 μg/ml killed the cells. In related research we have also shown that AMSA causes a 50% inhibition of partially purified DNA and RNA polymerases when assayed in vitro at drug:template DNA ratios of about 1:2.

By lysing cells directly on alkaline sucrose gradients prior to centrifugation, it has been possible to follow the incorporation of labeled precursors into DNA, to test the effects of various substances on the integrity of prelabeled DNA, or to measure the time course of repair following damage of DNA. These methods have been applied to mouse L1210 leukemia cells by Friedman et al. (5) to identify 3 distinct phases of chain growth in DNA replication and by Spataro and Kessel (11) to study an apparent degradation of DNA induced by camptothecin or acriflavine. This paper reports observations on alterations in DNA sedimentation profiles caused by administering AMSA to mice bearing L1210 cells or to L1210 cells in tissue culture medium.

MATERIALS AND METHODS

Materials. AMSA (provided by the Division of Cancer Treatment, National Cancer Institute) is an orange-red powder with limited solubility in water. Drug solutions were prepared fresh for each experiment by dissolving up to 1 mg/ml in dimethyl sulfoxide and diluting in growth medium or 0.9% NaCl solution as required. Controls were prepared similarly with dimethyl sulfoxide without drug. Some experiments used [9-14C]AMSA (specific activity, 11.4 Ci/mol; obtained from the National Cancer Institute).

Growth and Treatment of L1210 Cells. Mice used in these experiments were the DBA/2 strain (obtained from TIMCO, Houston, Texas) weighing approximately 20 g. L1210 tumor (LE34) cells were originally obtained from the National...
Cancer Institute and were transplanted by injection of a 0.25-ml volume of ascitic fluid diluted in Krebs-Ringer's solution to contain about 3 x 10^6 cells. Cells used in these experiments were grown for 3 days. Except when indicated otherwise cells were labeled in vivo with [3H]dTdThd (specific activity, 19 Ci/mmol) (New England Nuclear, Boston, Mass.) by i.p. injection of 20 μCi, diluted in sterile 0.9% NaCl solution; 18 to 20 hr before cells were harvested. This is referred to as long-term labeled DNA. For in vitro labeling or drug treatment, cells were collected from tumor-bearing mice in 1.5 ml of 137 mM NaCl-3 mM KCl-6 mM phosphate, pH 7.2, and mixed immediately in Roswell Park Memorial Institute Tissue Culture Medium 1630 diluted so that about 1 x 10^6 cells were suspended in 2 ml of medium augmented with 100 μg of gentamicin, 10% fetal calf serum, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.3 to 7.4). Cells were incubated with gentle shaking at 37° in capped 30-ml culture tubes.

Cell Lysis and Gradient Analysis. Cells were collected by centrifugation at 200 x g for 5 min, washed twice in at least 50 times the packed cell volume of 0.15 M NaCl-0.015 M sodium citrate, and finally suspended in the same solution so that 0.3 ml contained 0.5 to 1.0 x 10^6 cells as determined by analysis in a Coulter cell counter.

Neutral and alkaline lysis and gradient procedures were modifications of methods described by Cox et al. (4), Michael et al. (9), and Spataro and Kessel (11). The neutral lysing content 0.3% NaCl, 0.03 mM EDTA, and 0.1 M Tris-HCl (pH 7.8), and 1% sodium deoxycholate. The alkaline lysing content 0.19 M NaOH, 0.01 mM EDTA, and 0.5% sodium deoxycholate. Sucrose gradients were 5 to 20% in sucrose. In addition neutral gradients contained 1 M NaCl, 0.02 M Tris-HCl (pH 7.8), and 0.01 mM EDTA; alkaline gradients contained 1 M NaCl, 0.19 M NaOH, and 0.01 mM EDTA. Each profile presented represents a typical result from at least 3 experiments with similar results unless otherwise noted.

For experiments testing neutral, DNA-denaturing lysing conditions prior to centrifugation on neutral sucrose gradients, we used a medium containing 0.1 M Tris-HCl (pH 7.8), 30 mM EDTA, 0.3% NaCl, and 1% deoxycholate in 70% deionized formamide. An alternative denaturation procedure was also used in which cells lysed in the neutral medium described above were heated to 83° for 5 min and rapidly cooled prior to centrifugation.

For sedimentation analysis 0.25-4 inch cellulose nitrate tubes were used, and 0.3 ml of cell suspension was pipetted onto 0.5 ml of lysing solution layered over 14 ml of a 5 to 20% sucrose gradient resting on a 1-ml cushion of 2.3 M sucrose. Lysis was carried out at room temperature for 20 min and was followed by centrifugation for 18 hr at 15,000 rpm with the use of a Beckman SW25.3 rotor in an L2-65 centrifuge run at 20°. Fifteen or 16 fractions of 1.2 ml each were collected with an ISCO Model 640 fractionator by pushing 2.5 M sucrose, marked with blue dextran, into the bottom of the tube to displace the gradient upward. The dense sucrose cushion was found in Fractions 14 to 16. The samples were chilled to 0 to 2°, and 0.5 ml of 20% TCA containing 4% sodium pyrophosphate was added to each sample. Precipitates were collected on cellulose acetate filters 10 min later and washed 3 times with 5 ml of ice-cold 5% TCA-1% PPi. After drying, filters were placed in 5 ml of Permafluor solution (Packard Instrument Co., Downers Grove, Ill.), and radioactivity was determined on either a Packard 3004 or a Nuclear-Chicago Mark I scintillation spectrometer. The label in each fraction was calculated as percentage of the total acid-precipitable label recovered.

Approximate sedimentation values were calculated by the tables of McEwen (8) or, for values between 14 and 53S, by comparison with [3H]DNA markers (λ phage DNA, 40S on alkaline and 33S on neutral gradients) (Bethesda Research Laboratories, Inc., Bethesda, Md.). When either neutral or alkaline solutions were used for lysis, no whole cells could be found by microscopic examination 30 sec after mixing, nor could any cell counts be obtained with the Coulter counter within 10 sec of mixing the lysing solutions and a cell suspension.

Preparation of DNA. DNA was prepared from labeled L1210 cells by 2 methods. In the first, L1210 cells were processed as described above for neutral gradient analysis, except that after the cells were suspended in lysing solution they were incubated at room temperature for approximately 30 min, made 1 M in NaCl solution, and gently shaken for 30 min with an equal volume of a 1:1 solution of phenol in chloroform. The supernatant, following a 10,000 x g spin for 10 min, was treated with an equal volume of ethanol. The resulting stringy precipitate was collected, washed twice in 70% ethanol, and dissolved in 15 mM NaCl-1.5 mM sodium citrate. RNA was not removed from this solution; the nucleic acids were subsequently subjected to analysis in alkaline gradients for 18 hr at 20°, which hydrolyzed the RNA. In the second method labeled cells were subjected to the alkaline sucrose gradient procedure, and the fraction immediately above the dense sucrose cushion was collected and diluted 5-fold in 0.1 M phosphate buffer (pH 7.0). These solutions were incubated with AMSA to test direct effects of the drug on DNA. When nuclei were to be tested, they were prepared by lysis of L1210 cells with stirring in isotonic sucrose containing 3 mM MgCl2 and 0.1% Triton-X, followed by centrifugation through 1.4 M sucrose-3 mM MgCl2.

RESULTS

Cell Studies. Although denaturing conditions are used to lyse cells and sediment heavy DNA, the extent to which which this treatment separates proteins from the DNA has been disputed; the labeled material present in sucrose gradient fractions to some extent may be DNP as well as DNA. In a few experiments where L1210 cells were labeled with [3H]leucine, we found virtually all the label in the top 2 fractions of alkaline gradients.

Following a determination of the characteristics of dThd incorporation into L1210 cell DNA under our assay conditions, experiments on the effects of AMSA on DNA sedimentation profiles were conducted to determine (a) the effects of various doses of AMSA on prelabeled DNA, (b) the ability of cells to recover a normal pattern after exposure to AMSA, and (c) the effect of the presence of AMSA before or during DNA synthesis in comparison with the effect on prelabeled DNA.

The time course of in vivo incorporation of [3H]dTdThd into
DNA analyzed by our procedure on alkaline sucrose gradients was essentially the same when cells were exposed to the label either in vivo or in tissue culture (data not shown). The pattern of incorporation was similar in all respects to that shown by Friedman et al. (5). Analyses at 10, 20, and 30 min showed that a large proportion of the label was found in DNA sedimenting broadly in the 30S region, with less than 20% found as “heavy” DNA sedimenting into the dense sucrose cushion. By 1 hr 60% of the label was found in the heavy DNA. By 2 and 6 hr, 85 and over 90%, respectively, of the label sedimented in this heavy DNA region. Labeling for 16 or 24 hr produces a pattern identical with that for 6 hr. Centrifugation of such cell lysates for 5 instead of 15 hr revealed that the heavy DNA material sedimented in a fairly compact peak with a maximum at 180S; thus, DNA sedimenting to the cushion (Fractions 13 to 16) on standard analyses will be referred to as >170S or “heavy” DNA.

When cells containing DNA that had been labeled in vivo for 6 hr or longer (usually 18 to 20 hr) were exposed to AMSA for 30 min, analysis of DNA sedimentation showed a decrease in or complete absence of >170S DNA, with the appearance of label in the 30S region of the gradient (Chart 1; Table 1A). Doses of AMSA in the range of 100 μg/mouse (5 mg/kg) in vivo or 10 μg/ml in vitro brought about an almost complete transfer of label to the 30S region, with a low but significant degradation apparent at 0.25 μg/ml in vitro and 25 μg/ml in vivo. Degradation was detectable in treatment periods as short as 10 min (data not shown). Successively higher concentrations of AMSA tend to cause somewhat decreasing sedimentation values of the degraded DNA. However, higher doses of drug or longer exposure times did not produce large proportions of DNA sedimenting at the top of the gradient (data not shown). Furthermore, we found that this pattern remained essentially the same when cells were lysed during exposure for periods from 20 min to 5 hr before beginning sedimentation.

If sedimentation analyses were performed at increasing intervals after an acute exposure to AMSA, the pattern tended to return toward one resembling the control (Table 1B); for example, 6 hr after a 30-min exposure 30% of the label was again associated with >170S DNA. However, we have not observed complete recovery even as long as 24 hr after exposure. Essentially the same results were observed following in vivo exposure to AMSA, although we have not

### Distribution of DNA in various gradient fractions

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### Table 1

**Chart 1.** Effect of AMSA for 30 min in vivo on sedimentation of prelabeled L1210 DNA in alkaline sucrose gradients. AMSA at the doses shown below was injected i.p. into tumor-bearing mice that had received labeled dThd 18 hr previously. Cells were harvested after 30 min of treatment and were lysed on top of the alkaline 5 to 20% sucrose gradient, the tubes were centrifuged for 18 hr at 15,000 rpm, and acid-insoluble label in the fractions was measured as described in “Materials and Methods.” Fractions 14 to 16 represent the dense sucrose cushion, corresponding to sedimentation values greater than 170S. The vertical lines indicate the 1.2-ml fractions collected, and the symbols represent the cpm in each fraction, expressed as percentage of the total cpm recovered from the gradient. O, no AMSA (total cpm, 1704); A, 25 μg of AMSA per mouse (total cpm, 209); B, 10 μg of AMSA per mouse (total cpm, 1085).
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...determined independently the time course of drug concentration systemically.

Since AMSA inhibited the incorporation of dThd into DNA, we examined the size distribution of the DNA synthesized during the period of drug action. Chart 2 summarizes the data from experiments in which L1210 cells in culture medium were treated for 60 min with AMSA and then allowed to incorporate labeled dThd for 30 min. Chart 3 shows the distributions when labeled dThd and AMSA were injected simultaneously into mice bearing L1210 cells and the tumor was harvested after 90 min. Both experiments indicate that rapidly sedimenting DNA is diminished or absent in cells treated with AMSA. These results signify that the drug either prevented formation of heavy DNA fractions or caused degradation of newly synthesized heavy DNA, but further experiments are necessary to distinguish between these possibilities.

**Drug Complex Studies.** For characterization of the interaction between AMSA and DNA that might be responsible for the observations above, experiments were performed to test whether (a) alkaline conditions were necessary to observe the apparent fragmentation of >170S to 30S DNA, (b) labeled AMSA could be found in association with cellular DNA, (c) exposure to light was necessary for 30S DNA formation, (d) the interaction and fragmentation in alkali would occur if nuclei or purified DNA were exposed to the drug, (e) cell metabolism influenced the extent of the effects observed, and (f) other DNA-binding drugs produced similar effects.

Neutral lysis and sucrose gradient conditions were compared with alkaline lysis and gradient conditions. The sedimentation patterns of DNA, labeled 18 hr previously, from both control and AMSA-treated L1210 cells are illustrated in Chart 4. It is apparent that a significant shift to lighter DNA is not evident when the DNA from AMSA-treated cells is centrifuged under neutral conditions. We found that either neutral lysis before alkaline gradient sedimentation or alkaline lysis before neutral sedimentation gave the same pattern as did alkaline lysis and alkaline sedimentation. The degraded DNA seen after exposure to an alkaline condition could result either from the denaturation of duplex DNA, which would reveal single-strand breaks produced in the DNA following interaction with AMSA, or, alternatively, from alkaline hydrolysis of a site not previously broken but altered in some manner (e.g., loss of a single base) by interaction with AMSA. To distinguish between these possibilities, we heated a neutral lysate to 83° for 5 min and cooled it rapidly before placing it on a neutral sucrose...
gradient; in a second approach we lysed the cells in a neutral denaturing solution containing 70% formamide, as was used by Abelson and Penman (1) to test for alkali-labile sites in camptothecin-treated DNA. Centrifugation after either of these treatments resulted in sedimentation profiles indistinguishable from those of controls without AMSA (data not shown). Some random breakdown occurred with the thermal denaturation independent of exposure to AMSA, but the neutral denaturing and nondenaturing lysing conditions produced identical sedimentation profiles. These results suggest that alkaline conditions are necessary for the manifestation of DNA fragmentation.

Using $^{14}$C-labeled AMSA and $^{3}$H-labeled DNA, we measured the association of these isotopes in gradient fractions. Cells treated with AMSA are characteristically stained orange-yellow, which is the most obvious indication that the drug complexes with cell components other than DNA. Chart 5 depicts the distribution of acid-insoluble AMSA in alkaline and neutral gradients in experiments in which DNA was prelabeled for 2 hr in vivo before an exposure to a high dose of AMSA in vitro (also for 2 hr). This analysis is made on the label associated with fractions precipitated and washed by TCA at 0-2°C. Under neutral conditions almost 40% of the acid-insoluble AMSA is associated with the rapidly sedimenting peak, and the remainder is found at the top of the gradient, which contains little DNA. Since both DNA and associated proteins may sediment together under neutral conditions, the AMSA may be complexed with either the DNA or the protein, or both. After exposure to alkaline conditions, less than 2% of the $^{14}$C appears in the sedimenting fractions, with almost all of it appearing at the top of the gradient. The AMSA present at the top may signify a complex of drug with other cell components. Since the same cell suspension was sampled for both neutral and alkaline analyses, the ratio of total acid-insoluble isotopes recovered from the gradients is significant ($^{14}$C:$^{3}$H was 1.16 for neutral and 0.69 for alkaline; see the legend to Chart 5). The decreased proportion of $^{14}$C to $^{3}$H after alkaline sedimentation indicates that alkaline treatment dissociates the [$^{14}$C]AMSA from a complex with heavy DNA or DNP and renders it into acid-soluble form.

The possibility that the reaction of AMSA with DNA might be activated by light was tested, but the effects of AMSA on the distributions of either newly synthesized or long-term labeled DNA, analyzed by both alkaline and neutral conditions, were the same whether the entire process was shielded from or exposed to room fluorescent light (data not shown).

For determination of whether a direct degradative action of AMSA on DNA could be observed, the heavy DNA band from an alkaline gradient was diluted and neutralized to pH 8.0, incubated for 30 min at 37°C with AMSA, and reanalyzed on an alkaline gradient. We were unable to obtain a control that had not been at least partially degraded. The data shown in Table 1C illustrate that direct treatment of this DNA with AMSA did result in further alteration of its sedimentation pattern; i.e., the >170S material was degraded to smaller sizes. In agreement with Ref. 4, DNA prepared by deproteinizing in phenol was extensively degraded. Little >170S material was detectable, and this DNA was not significantly altered by treatment with AMSA. When nuclei were prepared from cells prelabeled with dThd and sedimentation patterns were analyzed after incubation with AMSA, the patterns obtained indicated that the preparation itself produced slowly sedimenting DNA species (none greater than ~50S) and that exposure of nuclei to AMSA was not effective in further reducing the size of this DNA (data not shown).

A few additional experiments were performed to determine whether the effect of the drug on DNA required active cellular metabolism. Untreated cells containing DNA labeled 18 hr previously were lysed in either alkaline or neutral layers that contained various amounts of AMSA and were centrifuged into alkaline sucrose gradients 15 min later. Even at doses of 25 /µg of AMSA per ml in the lysing layers, no changes were detected in sedimentation pattern (data not shown). In another approach, to test for the presence of soluble active metabolites we cocultured cells with labeled DNA for 10 min with unlabeled cells previously exposed to 25 /µg of AMSA per ml for 1 hr, and the DNA sedimentation pattern of the mixed-culture lysate was analyzed, but again no differences were found (data not shown).

In a preliminary screening experiment, 3 other xenobiotics (ethidium bromide, acridine orange, and phosphine orange) were tested at doses of 25 /µg/ml in culture medium with L1210 cells for 30 min. Data from alkaline sucrose sedimentation patterns are listed in Table 1D. These compounds produce various toxic effects in cells, including cell clumping, which was most probably responsible for the variable radioactivities of L1210 cell DNA recovered from the gradients in this experiment. All of these compounds intercalate with DNA to some extent, and all were similar to AMSA in their effect on DNA in this analysis. Similar results were reported for acriflavine by Spataro and Kessel (11).
DISCUSSION

These data give indirect evidence that the observed effects of AMSA on DNA are associated with the antitumor activity manifested by AMSA. The doses of AMSA used in vivo to study the effects detailed above are one-tenth of the single therapeutic dose found effective in increasing the average life span of mice bearing L1210 leukemia transplants. The concentrations used in vitro are 200 times the dose of AMSA that, when present continuously, produces a 50% cell death for L1210 cells grown in culture. The dose of drug that results in the complete loss of heavy DNA in alkaline sucrose gradient sedimentation patterns is approximately the same as that which produces 60 to 70% inhibition of dThd incorporation into L1210 cell DNA (125 Âµg versus 150 Âµg/mouse in vivo and 10 Âµg versus 4 Âµg/ml in culture medium). The data on the total radioactivity present in the gradients do not quantitatively reflect specific activities of the DNA at various doses of AMSA because the number of cells loaded on each gradient and the extent of prelabeling varied from 1 experiment to another. However, we have confirmed in 2 ways in our experiments that AMSA inhibits dThd incorporation. In 1 procedure L1210 cell DNA was labeled with [3H]dThd for 18 hr in situ; then the cells were harvested and placed in growth medium with [14C]dThd alone or in combination with 10 Âµg of AMSA per ml for 1.5 hr. The C:3H ratio was 30% lower in the drug-treated cells (in these cells 85% of the 3H appeared in the 30S region of the gradient, as anticipated from previous experiments). In a second approach cells were exposed in tissue culture medium to various doses of AMSA and then given [3H]dThd for 30 min; aliquots were analyzed for DNA (2) and label. Decreases of 37% at a level of 10 Âµg/ml and of 53% at 25 Âµg/ml were observed in DNA.

Because limited DNA fragmentation and the breakup of the AMSA-DNA (or AMSA-DNP) complex have been detected only after alkaline treatment, an actual in vivo chain reaction of dThd incorporation into L1210 cell DNA (125 Âµg versus 150 Âµg/mouse in vivo and 10 Âµg versus 4 Âµg/ml in culture medium) is not necessarily explain the inhibition of DNA synthesis observed during short-term treatment of cells with AMSA. We found that AMSA does directly inhibit the action in vitro of DNA polymerase, although the drug:DNA ratios required for inhibition appeared to be rather high (0.5) to be relevant to the action of AMSA on intact cells. This conventional assay for DNA polymerase utilizes denatured DNA as template and hence may not reflect true replication in vivo. In addition, we have observed that treatment of L1210 cells with AMSA also causes an increase in activity of nuclear polyadenosine diphosphate-ribose synthetase (results to be reported separately). Miller (10) has shown that DNases and other agents that fragment DNA stimulate polyadenosine diphosphate-ribose synthesis and believes that this process may be involved in repair of DNA. Further work to establish the interrelationships of the alkaline fragmentation, replication, and repair of DNA as functions of AMSA action and the cell cycle will be necessary to describe completely the cytotoxic action of the drug.

REFERENCES

Damage of DNA by AMSA


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