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ALKALI LABILITY OF DEOXYRIBONUCLEIC ACID THAT CONTAINS ADENINE ARABINOSIDE*

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Abstract—We have demonstrated previously that 9-β-D-arabinofuranosyl adenosine (ara-A), like 1-β-D-arabinofuranosyl cytosine (ara-C), incorporates exclusively into DNA, not RNA [Kufe *et al.*, *Cancer Res.* **43**, 2000 (1983)]. We have also demonstrated that (ara-C)DNA is degraded by alkali [Major *et al.*, *Biochem. Pharmac.* **31**, 861 (1982)], suggesting a structural instability of this abnormal nucleic acid. These findings have been extended by investigating the effects of various alkaline conditions on ara-A and (ara-A)DNA. The results indicate that the nucleoside was degraded under stringent conditions (0.4 M NaOH, 0.5 hr, 100°) and that a similar effect occurred following exposure of (ara-A)DNA. The results also indicate that milder alkaline conditions (0.4 M NaOH, 6 hr, 37°) resulted in strand scission of (ara-A)DNA at the 3′-carbon of the incorporated arabinosyl sugar without degradation of the ara-A moiety itself. These findings may be useful when attempting to purify (ara-A)DNA and confirm the structural instability of arabinosyl-containing DNA.

We have demonstrated previously that 1-β-D-arabinofuranosyl cytosine (ara-C) incorporates into leukemic cell DNA [1, 2]. These studies were performed under nondegrading conditions using cesium sulfate gradient analysis to distinguish between RNA and DNA. We have also demonstrated that the ara-C-containing DNA is labile to alkali at sites of ara-C incorporation [3].

More recently, we have investigated the incorporation of 9-β-D-arabinofuranosyl adenosine (ara-A) into leukemic cell nucleic acids [4]. These studies have shown that ara-A, like ara-C, incorporates exclusively into DNA, and not into RNA. These results are in contrast to published reports suggesting that ara-C and ara-A incorporate into both DNA and RNA [5–8]. This discrepancy may reflect methodologic differences.

In this report, we have studied the effect of alkaline digestion on the nucleoside [^{3}H]ara-A as well as [^{3}H]ara-A incorporated in DNA strands. The results demonstrate that (ara-A)DNA was degraded by alkali at a site adjacent to the 3′-carbon of the arabinosyl moiety. This finding suggests a structural instability of this abnormal nucleic acid and obviates the use of alkaline conditions in purifying ara-A-containing DNA.

MATERIALS AND METHODS

Cell culture. L1210 cells were grown as a suspension culture in Eagle's minimum essential medium (S-MEM) with 10% fetal calf serum (FCS), 100 units of streptomycin/ml, 100 μg penicillin/ml, 1% L-glutamine, and 0.05 mM 2-mercaptoethanol at 37° in a 5% CO_2 atmosphere.

Labeling of L1210 nucleic acids. The [adenine-2-^{3}H]ara-A (30 Ci/mmole, ICN, Irvine, CA) was purified prior to use by high pressure liquid chromatography (HPLC) using a 6% methanol elution [4]. This technique removes tritiated water and adenosine and results in approximately 99% purity of the [^{3}H]ara-A.

L1210 cells in logarithmic growth phase were washed twice with phosphate buffered saline (PBS) and were resuspended at 1×10^6 cells/ml in serum-free media. The cells were then pretreated for 30 min with 10^{-6} M deoxycoformycin (DCF) prior to adding 3 μCi/ml of [^{3}H]ara-A (30 Ci/mmole) for 6 hr. This concentration of DCF inhibits L1210 adenosine deaminase and prevents the deamination of ara-A [4, 9]. Cells labeled for analysis by cesium sulfate gradient centrifugation were also incubated with 10 μCi/ml of $H_3{}^{32}PO_4$ (carrier-free, New England Nuclear Corp., Boston, MA). The purification of cellular nucleic acids and cesium gradient analysis have been described previously [1].

Alkaline treatment of [^{3}H]ara-A and [^{3}H]ara-A-labeled L1210 DNA. The purified [^{3}H]ara-A was treated with 0.4 M NaOH for periods of 6 or 16 hr at 37°, or for 0.5 hr at 100°. Samples were then neutralized by the addition of 0.1 M Tris–HCl, (pH 7.8) and concentrated HCl.

DNA labeled with [^{3}H]ara-A was precipitated following Cs_2SO_4 centrifugation and then treated with 0.4 M NaOH for 6 or 16 hr at 37°. The acid-insoluble material was then precipitated with 0.7 N perchloric acid for 1 hr at 0°. Samples were spun at 15,000 *g* for 30 min in an Eppendorf Microfuge, and the pellet was redissolved in 0.01 M Tris–HCl (pH 7.8). The supernatant samples were neutralized with 4.0 M

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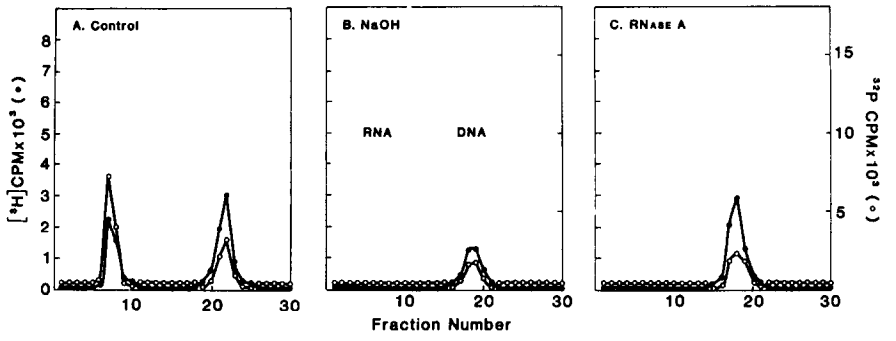

Fig. 1. Cesium sulfate density gradient analyses of [^{3}H]ara-A incorporation. L1210 cells in logarithmic growth phase at a concentration of 1×10^6 cells/ml were incubated with 10^{-7} M [^{3}H]ara-A and 10 μCi/ml of $H_3{}^{32}PO_4$ for 6 hr. The total cellular nucleic acids were purified and analyzed by cesium sulfate density gradient centrifugation (A). Aliquots were also treated with 0.4 M NaOH at 37° for 16 hr (B) or RNase A for 90 min at 37° (C). The tritium and ^{32}P radioactivity (cpm) banding in DNA regions of the gradients were as follows: (A) 6908, 6853; (B) 4154, 5409; and (C) 7275, 6706 respectively.

potassium bicarbonate, and the resulting potassium perchlorate precipitate was removed after incubation at 0° for 30 min. The acid-soluble and acid-insoluble fractions were then analyzed by HPLC either directly or following enzymatic digestion.

Enzymatic digestion of labeled L1210 nucleic acids. L1210 cellular nucleic acids were purified as described previously [1]. RNase A (Sigma Chemical Co., St. Louis, MO) digestion was performed at 0.5 mg/ml in 0.1 M acetate buffer (pH 5.0) for 90 min at 37°. The acid-soluble and -insoluble DNA fractions were then digested to either 5'-nucleotides, 3'-nucleotides or nucleosides [10–12].

HPLC analysis. The HPLC analyses were performed on a Varian 5020 high pressure liquid chromatograph (Varian Associates, Palo Alto, CA). Ion exchange chromatography and reverse phase chromatography were performed as previously described [2–4].

RESULTS

L1210 nucleic acids were labeled with [^{3}H]ara-A and ^{32}P after preincubation with DCF and analyzed by cesium sulfate gradient centrifugation (Fig. 1A). We have shown that, in the presence of DCF, the tritium radioactivity incorporated in DNA is exclusively ara-A and that the tritium banding in the RNA region of the gradient is exclusively adenosine [4]. Nucleic acid preparations were also treated with 0.4 M NaOH for 16 hr at 37° (Fig. 1B) or with RNase A for 90 min at 37° (Fig. 1C) prior to density centrifugation. Both alkali and RNase A digestion resulted in the loss of radioactivity banding in the RNA regions of the gradient. The alkali treatment also resulted in 58% reduction in the [^{3}H]ara-A and 21% reduction in the ^{32}P radioactivity banding in the DNA region of the gradient. A similar gradient profile was obtained when DNA was treated with 0.4 M NaOH for 6 hr at 37°. In contrast, no loss of tritium radioactivity was observed after a 6- or 16-hr alkali treatment of DNA labeled with deoxyadenosine.

The effects of various alkaline conditions on [^{3}H]ara-A were then investigated to determine susceptibility of the unincorporated nucleoside. Treatment with 0.4 M NaOH for 16 hr at 37° and analysis on reverse phase chromatography resulted in 55.4% degradation of [^{3}H]ara-A to a compound that comigrated with tritiated water on HPLC (Fig. 2). Approximately one-third of the tritium radioactivity, however, remained after lyophilization to remove tritiated water. This finding could be consistent with detection of an acyclic breakdown product of ara-A. Digestion with 0.4 M NaOH for 30 min at 100° yielded similar results. In contrast, milder digestion with 0.4 M NaOH for 6 hr at 37° resulted in no detectable degradation of [^{3}H]ara-A, as evidenced by the tritium radioactivity comigrating only with ara-A on reverse phase HPLC. Similar findings were obtained in six separate experiments. These results

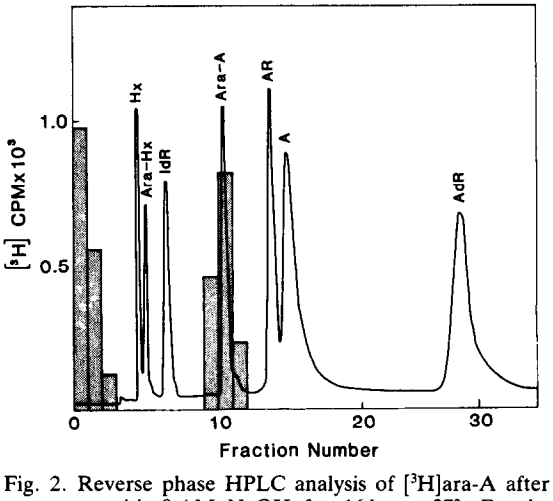

Fig. 2. Reverse phase HPLC analysis of [^{3}H]ara-A after treatment with 0.4 M NaOH for 16 hr at 37°. Results of three separate runs resulted in 54.7 ± 7.5% (mean ± S.D.) of the total radioactivity eluting in the initial three fractions.

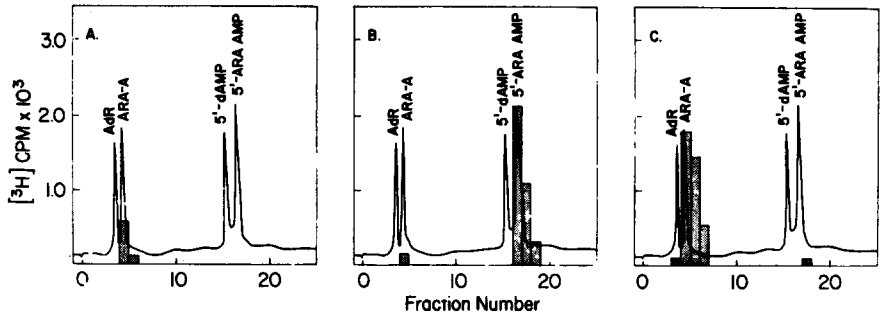

Fig. 3. Anion exchange HPLC analyses of acid-soluble [^{3}H]ara-A labeled DNA. (A) No enzymatic digestion. (B) After digestion with DNase I and snake venom phosphodiesterase. (C) After digestion with micrococcal nuclease and spleen phosphodiesterase.

suggest that mild alkaline conditions can be employed to degrade (ara-A)DNA without destroying the ara-A moiety itself.

The acid-soluble and -insoluble fractions obtained from treatment of (ara-A)DNA with 0.4 M NaOH for 6 hr at 37° were analyzed to determine whether ara-A incorporation results in strand scission under degrading conditions. The acid-soluble fraction contained approximately 50% of the total tritium radioactivity incorporated. Analysis of this fraction on anion exchange HPLC revealed that 9.7% (mean ± S.D.: 10.3 ± 0.7%) of the radioactivity was in the form of free [^{3}H]ara-A (Fig. 3A). This acid-soluble fraction was then digested with DNase I and snake venom phosphodiesterase. Anion exchange HPLC analysis of this digest showed that 3.1% (mean ± S.D. for three separate determinations: 1.8 ± 1.2%) of the [^{3}H]ara-A was located at the 5′-terminus of these DNA strands (Fig. 3B). The acid-soluble fraction was also digested with micrococcal nuclease and spleen phosphodiesterase. This approach resulted in comigration of 98.2% (mean ± S.D. for three separate determinations: 98.9 ± 0.94%) of the remaining radioactivity with ara-A (Fig. 3C). These findings suggest that mild alkaline treatment of (ara-A)DNA resulted in release of a free ara-A and of an acid-soluble DNA fragment which contained an ara-A moiety at the 3′-chain terminus.

The acid-insoluble fraction was analyzed in a similar manner. This fraction was initially digested with DNase I and snake venom phosphodiesterase (Fig. 4A). The results (three separate determinations) obtained indicate that less than 1% of the ara-A residues was present at the 5′-end of the DNA strand. Moreover, treatment with micrococcal nuclease and spleen phosphodiesterase demonstrated that 60% of the [^{3}H]ara-A residues was detectable at the 3′-terminus (Fig. 4B). The remainder of the radioactivity was thus present in internucleotide linkage. The unavailability of the 3′-ara-AMP standard prevented the inclusion of this marker in Figs. 3 and 4. However, previous experience [3, 13] indicates that 3′-monophosphates have retention times slightly longer than 5′-monophosphates when run under these conditions.

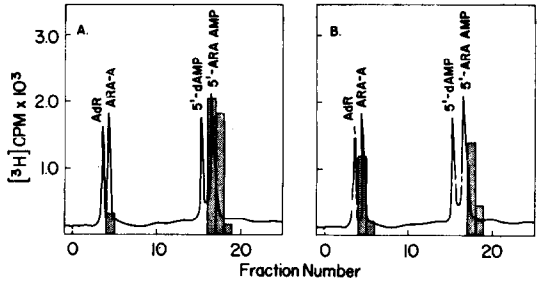

Fig. 4. Anion exchange HPLC analyses of acid-insoluble [^{3}H]ara-A labeled DNA. (A) After digestion with DNase I and snake venom phosphodiesterase. (B) After digestion with micrococcal nuclease and spleen phosphodiesterase.

These findings demonstrate that digestion of ([^{3}H]ara-A)DNA under mild alkaline conditions resulted in approximately 80–85% of the total [^{3}H]ara-A residues being at the 3′-chain terminus. This suggests selective strand breakage at the 3′ side of the incorporated ara-A moiety. In contrast, when control samples were treated in a similar manner but not subjected to alkali digestion, there was no detectable radioactivity in the acid-soluble fraction, and the acid-insoluble fraction contained approximately 5% of the [^{3}H]ara-A residues at the chain terminus position.

DISCUSSION

We have demonstrated previously that ara-C incorporates exclusively into cellular DNA [1–2], and that (ara-C)DNA is degraded under alkaline conditions [3]. Alkaline treatment of ([^{14}C]ara-C)DNA produced a radioactive material which was non-acid precipitable and which on both reverse phase and anion exchange chromatography was recovered in the early elution fractions. This labeled material was, therefore, not a structure with an intact pyrimidine ring and suggested that the cytosine ring incorporated in DNA as ara-C was labile to alkaline digestion. The recovery of intact ara-C residues after digestion of (ara-C)DNA also suggested cleavage of the phosphodiester bonds adjacent to these residues [3].

We have also demonstrated that ara-A incorporates specifically in DNA [4], and in the present studies we have explored the lability of (ara-A)DNA

under alkaline conditions. The data demonstrate that alkaline treatment of the unincorporated [^{3}H]ara-A residue resulted in partial exchange of the tritium label with water following exposure to 0.4 M NaOH for 16 hr at 37° or to 0.4 M NaOH for 30 min at 100°. In contrast, the nucleoside remained intact following a milder exposure to 0.4 M NaOH for 6 hr at 37°. The use of these milder conditions for degradation of (ara-A)DNA resulted in the release (acid-soluble fraction) of free ara-A and oligo nucleotides with ara-A residues detectable at the 3′-chain terminus. Further, the acid-insoluble fraction following alkaline treatment contained larger DNA strands that also had an increased proportion of ara-A residues at the 3′-terminus. These findings suggest that selective strand breakage can occur at the 3′-phosphodiester linkage. These findings are also similar to those obtained with ara-C [3].

The incorporation of arabinosyl derivatives in DNA thus provides potential sites for strand scission under alkaline conditions. The mechanism of strand breakage, however, is distinct from that obtained with hydrolysis of RNA in which a 2′:3′-phosphate intermediate is formed and results in the production of nucleoside 2′- and 3′-phosphates. Alkaline degradation of the arabinosyl compounds incorporated in DNA results in liberation of the arabinosyl-nucleoside and, therefore, loss of the phosphate group. This may result from an inability to form the 2′:3′-phosphate intermediate due to the transposition of the 2′-hydroxyl group on the arabinosyl sugar.

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