A 3′-5′ Exonuclease in Human Leukemia Cells

IMPLICATIONS FOR RESISTANCE TO 1-β-D-ARABINOFRANOSYLCYTOSINE AND 9-β-D-ARABINOFRANOSYL-2-FLUOROADENINE 5′-MONOPHOSPHATE

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A 3′-5′ exonuclease that excises the nucleotide analogs 1-β-D-arabinofuranosylcytosine monophosphate and 9-β-D-arabinofuranosyl-2-fluoroadenine 5′-monophosphate incorporated at 3′ ends of DNA was purified from the nuclei of: 1) primary human chronic lymphocytic leukemia cells, 2) primary and established human acute myeloblastic leukemia cells, and 3) lymphocytes obtained from healthy individuals. The activity of this nuclear exonuclease (exoN) is elevated approximately 6-fold in 1-β-D-arabinofuranosylcytosine-resistant leukemia cells as compared with drug-sensitive cells, and it differs between two healthy individuals and among three leukemia patients. exoN is a 46-kDa monomer, requires 50 mM KCl and 1 mM magnesium for optimal activity, and shows a preference for single-stranded over duplex DNA. Its physical and enzymatic properties indicate that exoN is a previously uncharacterized enzyme whose activity may confer resistance to clinical nucleoside analogs in leukemia cells.

ara-C and F-ara-A belong to a class of therapeutic compounds known as nucleoside analogs. Following their conversion to the active triphosphate form by cellular kinases, the analogs incorporate at 3′ ends of newly synthesized DNA strands to either decrease or terminate further chain elongation. Incorporation of either ara-CTP or F-ara-ATP into DNA is a critical event for the cytotoxic effect in the target cell (1, 2). ara-C is currently the most widely used drug for the treatment of acute myeloblastic leukemia (AML), whereas F-ara-A 5′-monophosphate (F-ara-AMP) is the agent of choice for treating chronic lymphocytic leukemia (CLL) (3, 4). However, clinical drug resistance limits the efficacy of these compounds (3, 4). In vitro, resistance to ara-C has been linked to reduction either in its transport or its metabolism and to increased dCTP pools, all of which result in decreased incorporation of ara-CTP into DNA (7–11). The underlying mechanism(s) of resistance to F-ara-AMP is not well characterized although sensitivity of cultured leukemia cells is directly related to the extent of incorporation of F-ara-ATP into DNA (12, 13).

Replicative DNA repair could impact on the extent to which ara-CTP and F-ara-ATP are incorporated into DNA in leukemia cells. Nuclear DNA polymerases δ and ε possess intrinsic 3′-5′ exonuclease activities to repair mispairs that arise when these polymerases insert an incorrect nucleotide at the 3′ end of the replicating DNA strand. Theoretically, these integral exonucleases could cleave out ara-CMP and F-ara-AMP incorporated into DNA in leukemia cells; however, previous studies have shown that they are generally inefficient at removing therapeutic nucleotide analogs from DNA (12, 14–17). In addition to DNA polymerase-associated exonucleases, unassociated 3′-5′ exonucleases have been described in mammalian cells. These include 3′-5′ exonucleases purified from whole cell and cytoplasmic extracts of human cells and from nuclear extracts prepared from animal cells (18–23). More importantly, some of these nucleases are proficient at excising a variety of nucleotide analogs from DNA in vitro (18–22).

It is conceivable that unassociated 3′-5′ exonucleases remove ara-CMP and F-ara-AMP residues from DNA to counteract the therapeutic effect of these drugs in leukemia cells. Accordingly, clinical samples from leukemia patients were examined for the existence of a putative exonuclease on substrates designed to resemble analog-terminated DNA in treated cells. Using a sequencing-gel exonuclease assay on DNA terminated with either ara-CMP or F-ara-AMP, a 3′-5′ exonuclease (exoN) was purified from nuclear extracts processed from one CLL patient, three AML patients, two AML cell lines, and two healthy individuals. The levels of exoN activity were compared between ara-C-resistant and drug-sensitive AML cells and in normal cells. The physical characteristics determined for exoN as well as its behavior on analog-terminated DNA indicate that it is a previously uncharacterized exonuclease.

EXPERIMENTAL PROCEDURES

Materials—ara-C was purchased from Sigma, and F-ara-ATP was a generous gift from Dr. William Parker (Southern Research Institute, Birmingham, AL). Phage M13mp18 (+) DNA and M13mp19 (+) DNA were purchased from Amersham Pharmacia Biotech and Life Technologies, Inc., respectively. The anion-exchanger diethylaminoethyl cellulose (grade DE52) was purchased from Whatman, and S-Sepharose cation-exchange resin was purchased from Sigma.

Primary and Cultured Leukemia Cells—Frozen cells from the two continuous lines AML-2 and AML-5 and from three AML patients were all generously provided by Dr. Mark Minden (Ontario Cancer Institute, Toronto, Ontario, Canada). The cell lines were originally established from peripheral blast cells of an untreated AML patient as described elsewhere (24), and AML-5 cells are 9-fold more resistant to ara-C than AML-2 cells (25). The established and primary AML cells were cultured in α-minimal essential medium supplemented with 10% heat-inacti-
FIG. 1. Purification of a 3′-5′ exonuclease from the nuclei of primary CLL cells. A, exonuclease profile of protein fractions eluted from an S-Sepharose column. Exonuclease activity was assayed on 3′-F-ara-AMP-terminated 19-mers in DNA duplexes for 60 min at 37 °C as described under “Experimental Procedures.” The reaction in lane L was performed with unbound protein from the preceding DE52 chromatography, whereas that in lane C was incubated for 60 min at 37 °C in the absence of enzyme. B, S-Sepharose fraction 20 was tested for removal of 3′-F-ara-AMP from duplex DNA for the indicated times at 37 °C, and the reaction products were quantified by densitometry as outlined under “Experimental Procedures.” The percentage of excised 3′-F-ara-AMP was plotted as a function of time.

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vated fetal bovine serum at 37 °C in 5% CO2 (AML-5 cells were grown in 10% medium conditioned by 5637-CM bladder carcinoma cells). Each cell line has a doubling time of approximately 14 h.

Clinical blood samples from a previously untreated patient diagnosed with CLL (20 ml) and from two healthy individuals (50 ml/volunteer) were collected and immediately processed for the isolation of lymphocytes using Ficoll density centrifugation as described previously (26). The number of viable lymphocytes obtained was 2 × 109 for the patient and 5 × 109 for each healthy volunteer.

Exonuclease Assay—Exonuclease activity was tested using a sequencing gel assay as detailed elsewhere (27). Briefly, 3′-terminated DNA substrates were prepared from an 18-mer with the following sequence: 5′-GTA AAA CGA CGG CCA GTG-3′.

This DNA primer is complementary to region 6291–6308 either on M13mp18 (+) phage DNA when terminated with F-ara-AMP or on M13mp18 (+) phage DNA when terminated with ara-CMP. The primer was labeled at the 5′ end with [γ-32P]ATP (specific activity 6000 Ci/mmol), purified on a G25 Sephadex column, and terminated at the 3′ end with the appropriate analog in reactions with 1 unit/ml terminal deoxynucleotidyltransferase in 250 mM potassium cacodylate, 25 mM Tris-HCl, pH 6.6, 0.25 mg/ml heat-inactivated BSA, and either 1.5 mM cobalt chloride and 25 mM F-ara-AMP or 0.75 mM cobalt chloride and 25 mM ara-C-TP. The terminated primers were purified as described previously (22), and portions of each primer were annealed to a 3-fold molar excess of either M13mp18 (+) or M13mp19 (+) DNA. For normally terminated DNA substrates, a primer with the above sequence was synthesized (Life Technologies, Inc.) with either an additional 3′-dCMP or 3′-dAMP residue in the 19th position. The direction of nucleotide removal was determined by exoN was tested as described elsewhere (22).

Exonuclease assays were done in 10-μl reactions containing 50 mM Tris-HCl, pH 8.0, 1 mM MgCl2, 1 mM dithiothreitol, 0.1 mM heat-inactivated BSA, 0.04 mM of the appropriate primer (either single-stranded or annealed to its complementary phage DNA), and 1 μl of the appropriate protein fraction. Incubations varied from 15 to 180 min at 37 °C, and reactions were terminated by adding 4 μl of 95% formamide, 10 mM EDTA, and 0.025% bromphenol blue. Samples were denatured at 100 °C for 5 min, followed by rapid cooling on ice. Electrophoresis was performed on 15% polyacrylamide, 7 M urea sequencing gels. The reaction products, detected as bands of radioactivity on sequencing gels, were quantified by a Molecular Dynamics Densitometer, and the exonuclease activity was expressed as percentage of a 3′-terminal nucleotide analog (e.g. ara-CMP or F-ara-AMP) removed from DNA. One unit of exonuclease activity is defined as the amount of enzyme that removes 10% of ara-CMP from the 3′ end of duplex DNA in 10 min at 37 °C.

DNA Polymerase Assay—The assays were performed in 15-μl reactions with 20 mM Tris-HCl, pH 7.4, 8 mM MgCl2, 1 mM dithiothreitol, 0.5 mM EDTA, 0.15 mg/ml heat-inactivated BSA, 0.15 mg/ml activated calf thymus DNA, 50 μM each of dATP, TTP, and dGTP, 1 μM dCTP, 1 μCi of [α-32P]dCTP (specific activity 6000 Ci/mmol), and 2.5 μl of the appropriate protein fraction at 37 °C for 60 min. The reaction mixtures were spotted onto Whatman DE-81 filter disks; the disks were washed three times with 0.3 M NaCl and fixed with 95% ethanol. The incorporated dCTP was measured by liquid scintillation counting, and the DNA polymerase activity expressed in units, where 1 unit is defined as the amount of protein required to incorporate 1 nmol of dCTP into DNA in 60 min at 37 °C.

Purification of exoN—All steps were performed at 4 °C unless otherwise specified. Nuclear protein extracts were prepared from 1–2 × 109 AML-2, AML-5, or CLL cells, 5 × 109 primary normal cells, and 1 × 107 primary AML cells. These cells contain approximately 0.1 mg of protein/106 cells (e.g. 0.1 ± 0.2 mg/106 AML-5 cells and 0.09 ± 0.4 mg/106 AML-2 cells). Nuclei were isolated as described previously with modifications (28). Briefly, cells were rinsed twice in phosphate-buffered saline, cell viability was checked by trypan blue exclusion, and the cell number was determined. The cells were incubated for 10 min in Buffer A (10 mM NaCl, 1.5 mM CaCl2, 10 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM benzamidine hydrochloride (Benz HCl) at a ratio of 5 ml of Buffer A to 1 ml of cell pellet. Approximately 95% of the cells were lysed in a Dounce homogenizer with a loose-fitting pestle as judged by phase contrast microscopy. The nuclei were removed by successive centrifugations for 4 min at 1430 × g. The pellet was washed twice in Buffer A; resuspended in 10 mM Tris-HCl, pH 8.0, 5 mM β-mercaptoethanol, 1% Triton X-100, 1 mM PMSF, and 1 mM benzamidine hydrochloride (Benz HCl) (2.5 ml/1 × 109 cells); mixed for 20 min; and centrifuged at 12,000 × g for 30 min. The supernatant was adjusted to 0.6 M NaCl, 10% glycerol and loaded on a DE52 column equilibrated in Buffer B (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 mM dithiothreitol, 10% glycerol, 1 mM each PMSF and Benz HCl) and 0.6 M NaCl. The unbound nuclear protein fraction was dialyzed against Buffer B and loaded on a DE52 column equilibrated in Buffer B. Protein that bound to DE52 was eluted in a linear gradient (0–1 M KCl), and all fractions were assayed for excision of ara-AMP or F-ara-AMP from 3′ ends of terminated DNA. The bound (flow-through) protein fraction, which contained approximately 95% of total exonuclease activity, was loaded on an S-Sepharose column, and the protein was eluted in a linear gradient (0–1 M KCl). A single peak of exonuclease activity eluted at 0.39 M KCl for each normal, CLL, and AML preparation. However, due to limited availability of primary AML cells, exoN recovered from these cells after the S-Sepharose step was less stable as compared with exoN purified from (larger numbers of other primary and established cells used in this study.)
performed for 60 min at 37 °C in the absence of enzyme. B
(0.03 M volume (elution volume for blue dextran 2000), and
cellulose membrane in two changes of 50 mM Tris-HCl, pH 7.8, 25%
weight. The transferred exoN preparation was renatured on the nitro-
standards (Amersham Pharmacia Biotech) were similarly processed to
SDS-PAGE, followed by transfer of the protein to a nitrocellulose mem-
tronography system. The column had been calibrated with the following
ations for excision of 3′-ara-CMP from 19-mers for 60 min at 37 °C as
described under “Experimental Procedures.” The reaction in lane C was
for 60 min at 37 °C in the absence of enzyme. B, fraction 19
(0.05 μg) from S-Sepharose chromatography of AML-5 nuclear protein
was stained with silver nitrate following 10% SDS-PAGE. The migration
of molecular size standards is indicated on the left-hand side.
Hence, exoN activity in primary AML cells was examined in the flow-
through protein fraction from the preceding chromatography step on
DE52. Protein concentration was measured using the Bio-Rad assay.
The purity of exoN was assessed by: 1) quantifying excision of ara-CMP
or F-ara-AMP from DNA after each purification step by densitometry;
2) 10% SDS-PAGE followed by staining with silver nitrate. Fractiona-
tion to nuclei followed by chromatography on DE52 and S-Sepharose
typically resulted in 2600-fold purification of exoN from either primary
or established cells to a specific activity of approximately 7.7 × 105
units/mg of protein.

Molecular Weight of exoN—Gel filtration was performed with 200 μl
of AML-5 exoN recovered from chromatography on S-Sepharose. The
sample was loaded on a Superdex 200 HR 10/30 pre-packed column and
eluted in Buffer B and 0.35 M KCl using the fast protein liquid chro-
matography system. The column had been calibrated with the following
protein standards: ribonuclease A (13.7 kDa), chymotrypsinogen A (25
kDa), ovalbumin (43 kDa), albumin (67 kDa), and aldolase (158 kDa). A
standard curve of molecular weight versus the partition coefficient $K_{av}$
was plotted, where $K_{av}$ was calculated for each protein by Equation 1.

$$K_{av} = \frac{V_e - V_s}{V_t - V_s} \quad (\text{Eq. 1})$$

$V_e$ is the elution volume for a given protein standard, $V_s$ is the void
volume (elution volume for blue dextran 2000), and $V_t$ is the total bed
volume (29). The $K_{av}$ calculated for exoN was used to extrapolate its
molecular weight from the standard curve.

In situ protein renaturation of exoN from AML-5 cells was performed
according to a previously published procedure with several modifica-
tions (30). Briefly, 100 μl of S-Sepharose fraction 19 was loaded on 10%
SDS-PAGE, followed by transfer of the protein to a nitrocellulose mem-
brane using the Bio-Rad Mini-Protean II system. Rainbow protein
standards (Amersham Pharmacia Biotech) were similarly processed to
visualize the migration and transfer of proteins of known molecular
weight. The transferred exoN preparation was renatured on the nitro-
cellulose membrane in two changes of 50 mM Tris-HCl, pH 7.8, 25%
2-propanol, and 5 mM β-mercaptoethanol at room temperature for 30
min, followed by incubation in 50 mM Tris-HCl, pH 7.8, 25 mM KCl, 0.2
mg/ml heat-inactivated BSA, 5 mM β-mercaptoethanol, and 20% glyc-
erol for 18 h at 4 °C. The nitrocellulose area that contained the rena-
tured protein from fraction 19 was cut into 10 1–2-mm strips. Each strip
was tested for exonuclease activity in reactions with 0.4 nM 5′ end-
radiolabeled and 3′-ara-CMP-terminated 19-mers for 3 h at 37 °C. The
reactions (minus the nitrocellulose strips) were loaded on sequencing
gels, and reaction products were detected by autoradiography.

**RESULTS**

Identification of a 3′-5′ Exonuclease in the Nuclei of Primary
Leukemia Cells—Nuclear protein prepared from a CLL patient
was fractionated on DE52 anion exchange, and the eluted fractions
were tested for both exonuclease and DNA polymer-
ase activity. More than 90% of DNA polymerase activity bound
to DE52 and eluted at 0.16 M KCl (results not shown). In
contrast, approximately 95% of exonuclease activity that
claved F-ara-AMP from 3′ ends of DNA was detected in the
unbound protein fraction, which upon fractionation on S-Sepharose yielded one peak of exonuclease activity that
eluted at 0.39 M KCl (Fig. 1A). The excision of F-ara-AMP
was optimal in reactions with fraction 20, as indicated by compar-
the relative ability of the eluted fractions to convert analog-
terminated 19-mers to shorter DNA fragments that migrated

**Fig. 2. exoN in ara-C-resistant and ara-C-sensitive AML cells.** A, exoN peaks obtained after S-Sepharose chromatography of nuclear
protein extracts from ara-C-resistant AML-5 or drug-sensitive AML-2
cells. The exonuclease peaks were identified by assaying eluted frac-
tions for excision of 3′-ara-CMP from 19-mers for 60 min at 37 °C as
indicated under “Experimental Procedures.” The reaction in lane C was
performed for 60 min at 37 °C in the absence of enzyme. B, fraction 19
(0.05 μg) from S-Sepharose chromatography of AML-5 nuclear protein
was stained with silver nitrate following 10% SDS-PAGE. The migration
of molecular size standards is indicated on the left-hand side.

**Fig. 3. Removal of ara-CMP by exoN purified from ara-C-re-
sistant and ara-C-sensitive AML cells.** A, S-Sepharose fraction 19
from AML-5 and AML-2 cells was assayed for excision of 3′-ara-CMP
from DNA duplexes at 37 °C for the indicated times as outlined under
“Experimental Procedures.” B, the excision products were quantified
by densitometry and the percentage of excised 3′-ara-CMP was plotted
as a function of time. Each point represents the mean ± standard devia-
tion for exoN activity obtained from three separate preparations of
nuclear protein extracts from AML-5 (●) and AML-2 (□) cells.
that range in size from approximately 30 to 130 kDa. The activity of exoN is increased in ara-C-resistant AML cells. Time-course studies of ara-CMP excision from DNA duplexes confirmed that exoN from AML-5 cells removes ara-CMP more efficiently than exoN from AML-2 cells (Fig. 3A). This result was reproduced three times with separate preparations of AML-5 and AML-2 nuclear protein (Fig. 3B). Under linear reaction conditions (initial 15 min), exoN from AML-5 cells cleaved approximately 6-fold more ara-CMP as compared with exoN from AML-2 cells. In contrast to exoN activity, similar levels of DNA polymerase activity were recovered from AML-5 and AML-2 cells (36 and 37 units, respectively).

exoN Activity in Primary Normal and AML Cells—Lymphocytes from two healthy individuals were processed concurrently to obtain two S-Sepharose-purified preparations of nuclear exoN. The exonuclease activity was tested on 3'-ara-CMP- and 3'-F-ara-AMP-terminated DNA, and the percentage of analog removed was quantified and plotted as a function of time (Fig. 4, A and B). A comparison of the initial excision rates revealed that, after 30 min, exoN from volunteer 1 excised approximately 5 times more 3'-ara-CMP and 3 times more 3’-F-ara-AMP as compared with exoN from volunteer 2. At all other time points tested, exoN purified from volunteer 1 removed approximately 2-fold more of each nucleotide analog from DNA as compared with exoN purified from volunteer 2.

Finally, nuclear extracts from three AML patients were fractionated on DE52, and the partially purified exoN in the unbound protein fraction was tested for removal of ara-CMP from duplex DNA. After 60 min at 37 °C, exoN from ara-C-resistant AML patients 1 and 2 removed 40% and 46% of ara-CMP, respectively, as compared with 30% of ara-CMP excised by exoN from the third, ara-C-sensitive patient.

Properties of exoN—An aliquot of exoN purified from AML-5 cells was analyzed by gel filtration. A single peak of exonuclease activity was detected in fractions 36–39, and its calculated \( K_{av} \) of 0.443 corresponds to a 45.3-kDa protein on the standard curve (Fig. 5A).

In situ protein renaturation studies were performed to determine the oligomerization status of exoN. A portion of S-Sepharose-purified exoN from AML-5 cells was loaded on 10% SDS-PAGE, followed by transfer of the protein to a nitrocellulose membrane. The migration of exoN relative to protein standards of known molecular weight was deduced by cutting the nitrocellulose into 1–2-mm segments and testing each segment for renatured exonuclease activity on sequencing gels. A renatured protein in segment 6 excises ara-CMP from DNA and co-migrates with the 46-kDa protein standard on SDS-PAGE (Fig. 5B).

Table I summarizes the properties of exoN. It is a 3'-5' directed exonuclease that preferentially cleaves nucleotides from single-stranded DNA, and is at least as active on nucleotide analog-terminated DNA as on 3' ends of DNA that contain naturally occurring nucleotides. exoN activity requires 50 mM KCl and 1 mM magnesium for optimal activity, whereas it is completely inhibited when either zinc or calcium replaces magnesium in the exonuclease reactions. exoN can utilize manganese instead of magnesium; however, its activity is decreased in the presence of the former divalent metal.

**DISCUSSION**

The potency of ara-C and F-ara-AMP is dependent upon their incorporation at the 3' ends of replicating DNA in tumor cells (1, 2). We identified a 3'-5' exonuclease in human cells, which efficiently removes ara-CMP and F-ara-AMP from synthetic substrates that mimic analog-terminated DNA. exoN was purified from nuclei derived from four leukemia patients and two healthy blood donors, and this suggests that it is a ubiquitous enzyme that may reverse incorporation of therapeutic nucleoside analogs into DNA in leukemia cells.
The activity of a DNA polymerase-unassociated 3′-5′ exonuclease might be important in cells treated with F-ara-AMP, as this analog was previously shown to inactivate the 3′-5′ exonuclease subunit of DNA polymerase ε (17). exoN removes increasing amounts of F-ara-AMP as a function of time, suggesting that it is not inactivated by the excised nucleotide analog (Figs. 1B and 4B). Thus, the activity of exoN could be a critical determinant of the potency of F-ara-AMP in CLL cells.

The idea that resistance to nucleoside analogs is secondary to exoN activity is supported by our demonstration that exoN purified from drug-resistant cells excises more ara-CMP from DNA than exoN purified from drug-sensitive cells. Additionally, exoN activity varies between two healthy volunteers and among three AML patients. These findings provide preliminary evidence for the existence of variations in constitutive exoN activity among individuals and for the induction of this activity by ara-C. These attributes could partially explain both de novo and acquired resistance to nucleoside analogs among leukemia patients (5). Other previously characterized mechanisms of resistance include decreased transport and/or decreased phosphorylation of ara-C to its active metabolite (7–11). The activity of exoN may constitute another important mechanism of resistance to clinical nucleoside analogs in leukemia cells.

A number of DNA polymerase-unassociated 3′-5′ exonuclease...
ases have been described in human cells (18–20, 22, 23, 30–34); however, we detect one major activity that removes each ara-CMP and F-ara-AMP from 3’ termini of DNA. Additionally, certain enzymatic and physical properties of exoN, especially its behavior during chromatography, magnesium requirement, molecular weight, and oligomerization status, distinguish it from other 3’-5’ exonucleases, and this suggests that we have identified a previously uncharacterized protein.

The up-regulation of exoN activity in resistant AML cells might occur either at the transcriptional or translational level; alternatively, it may result from a mutation in the exoN gene. The up-regulation of exoN activity in resistant AML cells (19) might occur either at the transcriptional or translational level; alternatively, it may result from a mutation in the exoN gene.

| Molecular mass (kDa) | 46 |
|---------------------|----|
| Oligomerization status | Monomer |
| MgCl₂ (mM) for optimal activity | 1 |
| MnCl₂ (mM) for optimal activity | 0.1 |
| KCl (mM) for optimal activity | 50 |
| Direction of nucleotide removal from DNA | 3’→5’ |
| Preferential excision of single-stranded DNA | 1.5 |
| Preferential excision of ara-CMP | 1 |
| Preferential excision of F-ara-AMP | 3 |

a The activity of exoN (purified from either AML-5 or AML-2 cells) was determined in reactions with the appropriate 5’ end-radiolabeled DNA substrate as outlined under “Experimental Procedures.”

b The direction of exoN excision was examined in reactions with 3’ end-radiolabeled DNA as outlined under “Experimental Procedures.”

c Ratio of removal of ara-CMP versus dCMP or F-ara-AMP versus dAMP from 3’ ends of DNA.

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