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A rapid and sensitive GC-MS/MS method to measure deuterium labeled deoxyadenosine in DNA from limited mouse cell populations

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Abstract

A rapid and sensitive GC-MS/MS method was developed to quantitatively measure low levels of DNA base deoxyadenosine (dA) and its isotopologues (e.g. dA M+1) from limited mouse cell populations. Mice undergoing allogeneic hematopoietic transplantation (AHSCT) received deuterated water at biologically relevant time intervals post AHSCT, allowing labeling of DNA upon cell division, which was detected as the dA M+1 isotopologue. Targeted mouse cell populations were isolated from lymphoid organs and purified by multi-parameter fluorescence activated cell sorting. Cell lysis, DNA extraction and hydrolysis were accomplished using available commercial procedures. The novel analytical method utilized a hydrophilic-lipophilic balanced sample preparation, rapid on-line hot GC inlet gas phase sample derivatization, fast GC low thermal mass technology, and a recently marketed GC-MS/MS system. Calibration standards containing dA and fortified with relevant levels of dA M+1 (0.25–20%) and dA M+5 (internal standard) were used for sample quantitation. The method employed a quadratic fit for calibration

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ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Author Contributions

D.F. and N.B. are joint first authors and contributed equally to the research. The manuscript was written through contributions from each author, with the final version of the manuscript being approved by all authors.

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of dA M+1 (0.25–20%) and dA, demonstrated excellent accuracy and precision, and had limits of detection of 100 fg on-column for the dA isotopologues. The method was validated and required only 20,000 cells to characterize population dynamics of cells involved in the biology of chronic graft-versus-host disease, the main cause of late morbidity and non-relapse-mortality following AHSCT. The high sensitivity and specificity of the method makes it useful for investigating *in vivo* kinetics on limited and important cell populations (e.g. T regulatory cells) from disease conditions or in disease models that are immune-mediated, such as diabetes, HIV/AIDS, arthritis, inflammatory bowel disease, and multiple sclerosis.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (AHSCT) is a curative therapy for patients with heritable disorders and malignancies, many of which are refractory to other treatments. Chronic graft-versus-host disease (cGVHD) is the main cause of late morbidity and non-relapse mortality after AHSCT,[1–5] with an estimated incidence of 50%, and as high as 80% in some cohorts. Little progress in treatment or prevention of cGVHD has been accomplished in the past 30 years; hence cGVHD presents an important unmet clinical need with limited understanding of its pathophysiology. T cells are known to mediate cGVHD, however little is known about their kinetics, despite the fact that current therapies target T cell proliferation.[1,3,4,6–8] T regulatory (Treg) cells are of particular interest because adoptive transfer of Treg cells has been shown to ameliorate cGVHD in pre-clinical and clinical studies.[6–8] Treg cell kinetics in pre-clinical models are not amenable to investigation with existing methods; hence, our GC-MS/MS method was developed to allow investigation of *in vivo* kinetics of this limited and important cell population.

Measurement of immune cell turnover has been previously studied in mice by utilizing bromodeoxyuridine, carboxyfluorescein diacetate succinimidyl ester, or tritiated thymidine.[9] However, each of these labeling methodologies is hindered by significant limitations and cannot be used in the clinical setting. The advantages of using stable deuterated isotopes such as deuterated water are: 1) lack of toxicity, 2) no affect of label on cell division, 3) gain of deuterium enrichment being directly related to cell division, and 4) applicability to the clinical setting.[9] Administration of deuterated water leads to incorporation of the deuterium moiety into deoxynucleosides of DNA through the *de novo* nucleotide synthesis pathway, the primary and constitutive pathway of nucleotide synthesis.[9] Busch *et al.*, who have been in the field for more than a decade, have hypothesized that the deuterium incorporates into the hydrocarbon bonds of the deoxyribose moiety.[9]

Prior publications evaluating stable deuterium enrichments into genomic DNA utilized analytical techniques such as single quadrupole GC/MS, LC/MS and GC-pyrolysis-IR/MS.[9–16] These techniques required processing more than 50,000 cells to quantitatively measure DNA deoxynucleosides (e.g. dA) and the low levels of deuterium enrichment found in the dA isotopologues (e.g. dA M+1) of proliferating cells, which precludes investigations of important lymphocyte populations such as Tregs. Past GC/MS methods also utilized various derivatization techniques (e.g. methylation, pentafluorobenzyl hydroxylamine, and pentane-tetraacetate), which were lengthy (i.e. heated incubation for hours) and hazardous (i.e. require fume hood).

In our method, the MethElute™ reagent rapidly derivatizes dA and its isotopologues in the heated GC injection port (termed on-line hot inlet gas phase sample derivatization). The MethElute™ derivatization approach is similar to Fox *et al.*[10]; however our method does not require the use of an organic modifier (e.g. dimethylformamide), or processing a large number of cells for analysis. Fox *et al.* also reported a sensitive LC/MS method, which did

not require sample derivatization; yet it required processing 125,000 cells for analysis. The GC-pyrolysis-IR/MS technique reported ultra-high sensitivity for measuring trace levels of deuterium enrichment into the dA isotopologues; however it required processing 1×10^7 cells for sample analysis.[16] In this article, we describe a novel GC-MS/MS method, which is rapid and sensitive for measuring dA and its isotopologues in DNA from mouse studies involving limited cell populations (i.e. Tregs). After cells were harvested from mouse tissues and sorted using fluorescence activated cell sorting (FACS), commercial kits were used to perform cell lysis, DNA collection and hydrolysis to the deoxynucleoside base pairs. The deoxynucleoside base pairs (e.g. dA) were purified and concentrated using a hydrophilic-lipophilic balanced (HLB) solid phase extraction (SPE), and derivatized rapidly in the heated GC injection port. Rapid chromatography was performed using a low thermal mass (LTM) GC oven module and LTM narrow-bore capillary column, which provided high component resolution and short analysis time (~4.5 min). To attain high method sensitivity and specificity, the method utilized positive chemical ionization (PCI), stable isotope internal standard (dA M+5, I.S.), and the multiple reaction monitoring (MRM) mode of tandem MS. As oxidative DNA adducts (e.g. 8-oxo-dA) have been implicated in several disease conditions (e.g. cancer), the method was set up to concurrently monitor for the 8-oxo-dA adducts.[17,18]

EXPERIMENTAL SECTION

Chemicals, Reagents, Antibodies, Commercial Kits, and Gases

Deuterium Oxide (D, 99.8%) was purchased from Cambridge Isotope Laboratories (Andover, MA). Methanol (high purity) was purchased from Honeywell Burdick & Jackson® (Muskegon, MI). Acetone (HPLC grade) was purchased from Fisher Scientific (Fairlawn, NJ). Phosphate buffered saline (PBS 1X, pH 7.4) was purchased from GIBCO (Invitrogen™, Grand Island, NY).

Molecular Biology Grade deionized water and ACK Lysis Buffer were purchased from Quality Biological, Inc. (Gaithersburg, MD). MethElute™ derivatization reagent (0.2 M trimethylanilinium hydroxide in methanol, (pH ≥ 10) was purchased from Thermo Scientific (Waltham, MA) and used as received. Sodium Chloride was purchased from JT Baker (Philipsburg, NJ). Commercial kits for cell lysis, Maxwell® 16 LEV Blood DNA kits, and DNA hydrolysis (EpiQuik™) were purchased from Promega (Madison, WI) and Epigentek (Farmingdale, NY), respectively. Oasis® HLB μElution plates (30 μM) were purchased from Waters Corporation (Milford, MA). Fixation/Permeabilization Concentrate (4X), Fixation/ Permeabilization Diluent (1X), Permeabilization Buffer (10X), and anti-mouse Foxp3 PE antibody (clone FJK-16s) were purchased from eBioscience (San Diego, CA).

Standard material of 2'-deoxyadenosine monohydrate (dA, purity > 99%), $^2H_2$-deoxyadenosine monohydrate (dA M+2 isotopologue, purity 99%), Albumin solution from Bovine Serum (30%), and DNA from human placenta (~90% w/w, sodium salt form) were purchased from Sigma-Aldrich (St Louis, MO). The $^{13}C_1$-deoxyadenosine monohydrate (dA M+1 isotopologue, purity > 98%), U-$^{15}N_5$-deoxyadenosine (dA M+5 isotopologue, purity 96–98%), and deuterium oxide (99.8% enrichment) were purchased from Cambridge Isotopes (Andover, MA). Standard material for measuring DNA oxidative adduct 8-oxo-deoxyadenosine (purity > 98%) was purchased from ZeptoMetrix Corporation (Buffalo, NY). Gases used for GC-MS/MS analysis were helium (Grade 5.5 purity), nitrogen (Ultra high purity), isobutane (Matheson 99.99% purity) and methane (Research Grade purity) and were purchased from Roberts Oxygen Company (Rockville, MD). Gas purifiers purchased from Agilent Technologies (Santa Clara, CA) were used to remove hydrocarbons and moisture from the GC-MS/MS gases.

Equipment and Workstation Software

The Becton-Dickinson (San Jose, CA) LSRII was used for FACS analysis and the Becton-Dickinson Influx was used for cell sorting. The Promega Maxwell® 16 system (Madison, WI) was utilized for cell lysis and DNA purification. The Eppendorf Thermomixer R used for DNA hydrolysis was purchased from Eppendorf North America (Hauppauge, NY). The extraction plate manifold used for Oasis 96-well plate HLB extractions was purchased from Waters Corporation (Milford, MA). The Savant DNA110 SpeedVac® used for concentrating samples was purchased from Thermo Fisher Scientific (Waltham, MA). The Agilent 7890A GC, LTM Series II Fast GC Module, 7000A GC-MS Triple Quadrupole, and 7693 Autosampler were purchased from Agilent Technologies, Inc. (Santa Clara, CA). For GC-MS/MS data acquisition and processing, Agilent MassHunter workstation software included GC-MS/MS Acquisition (Version B.05.02.1032), Qualitative Analysis (Version B.04.00) and Quantitative Analysis (Version B.05.00).

GC-MS/MS Calibration Standards, Internal Standard and QC Solutions

Supporting Information 1, GC-MS/MS Calibration Standards, Internal Standard and QC Solutions describes the preparation of calibration standards and QC solutions.

Deuterium Labeling of Proliferating Cells

The deuterium labeling protocol for mice involved an initial intraperitoneal (IP) bolus of 35 ml/kg of 0.9% (wt/wt) NaCl in 100% ^2H_2O, followed by continuous administration of 8% ^2H_2O in drinking water until cell harvest.[9] To measure cell proliferation, deuterium enrichment into the DNA of the cells of interest is compared to enrichment of a fully turned over reference population over a specified labeling period.

Mouse Protocol, GVHD Scoring and Cell Harvesting

Supporting Information 2, Mouse Protocol, GVHD Scoring and Cell Harvesting describes experimental conditions used for the mouse studies.

Flow Cytometry and FACS Analysis

Supporting Information 3, Flow Cytometry and FACS Analysis describes the experimental conditions used for Flow Cytometry and FACS analysis.

Cell Lysis, DNA Extraction and Hydrolysis

Commercial kits were available for extracting DNA from harvested cells, and to hydrolyze DNA into its deoxynucleoside base pairs. Briefly, the Promega Maxwell® 16 system utilizes detergent to lyse the cell membranes, extracts DNA using magnetic beads coated with cellulose, and wash steps to purify the DNA for downstream procedures (e.g. PCR). Highly purified DNA was obtained with typical yields ranging from 40–60%. After purified DNA was extracted from the cells, DNA hydrolysis was accomplished using a commercially available kit (Epigentek EpiQuik™). The kit contains DNA hydrolysis enzymes and digestion buffer, and was used as received. Following 2-hour incubation at 37°C and gentle mixing using an Eppendorf Thermomixer R, the kits typically provided linear DNA hydrolysis (75 to 600 ng) to its deoxynucleoside base pairs with efficiencies between 60–90% (Supporting Information, Figure 1).

Deoxynucleoside Purification and Derivatization

A HLB solid phase extraction was utilized to remove DNA hydrolysis buffer salts, sample water, and to concentrate the sample prior to GC-MS/MS analysis. The HLB procedure is listed in Supporting Information, Table 1. For sample reconstitution, 25 μl of the

MethElute™ derivatization reagent was pipetted directly into the autosampler microvial containing the sample residue, capped and vortex for 15 seconds. The method used an on-line hot inlet gas phase technique, which safely and rapidly derivatized dA, the dA isotopologues, and 8-oxo-dA.

GC-MS/MS Instrument Conditions

Supporting Information, Tables 2 and 3 lists final GC and MS instrument conditions used for sample analysis, respectively.

Computations

The goal of this method targeted 6 μM dA, which corresponds to ~150 ng genomic DNA extracted from ~20,000 mouse cells. The 6 μM dA target assumed 100% yield of genomic DNA extracted from the mouse cells, and 100% hydrolysis to the deoxynucleoside base pairs. MS multiple reaction monitoring mode using peak height response and Agilent MassHunter Quantitation software was used for component computations. For data analysis, calibration standards containing 6 μM dA, dA M+1 enrichments of 0.25 to 20%, and 2 μM dA M+5 (I.S.) were used for method calibration.

The dA calibration model utilized average response factors from standards containing 6 μM dA and the internal standard (2 μM dA M+5). For dA isotopologue computations (e.g. M +1), calibration was performed using Microsoft Excel 2010 software. Computations used a polynomial fit of X (i.e. dA M+1 %enrichment) versus Y (i.e. ratio of MS peak height response (M+1) / ((M0) + (M+1))). Excel calculated the polynomial fit parameters and determined the coefficient of determination (R^2). The rationale for using a ratio computation was to maintain consistency to investigators in the field using deuterated stable isotopes for their cell proliferation research.[9–13,15,16]

RESULTS AND DISCUSSION

GC-MS/MS Method Development and Optimization

Our use of a GC-MS/MS for detection and quantitation of dA and its isotopologues is the first reported in the literature. The GC-MS/MS is capable of multiple rounds of mass spectrometry, often separated by some form of molecule fragmentation. During MS operation, the first mass analyzer (Q1) can isolate one component from others that enter the mass spectrometer. The second mass analyzer (Q2) induces collision with an inert gas (e.g. nitrogen), which causes fragmentation via collision-induced dissociation (CID). The third mass analyzer (Q3) sorts the fragments produced by the CID. Multiple reaction monitoring is one mode of GC-MS/MS analysis, and provides the highest sensitivity and selectivity for trace level analysis. For this method, each component's MRM transition was chosen to provide the highest sensitivity and selectivity. For example, the MRM transition monitored for the PCI dA derivative was m/z 308 → 164.

The GC-MS/MS method was developed and validated for cell kinetics research. Although the methodology was used to measure levels of DNA deoxynucleoside base pair dA and deuterium enrichment of the dA M+1 isotopologue, it can also quantitatively measure the other dA isotopologues (e.g. M+2). For clarity throughout this article, deoxyadenosine, dA, and dA M0 are equivalent terms. When using deuterated water (^2H_2O) as the DNA enrichment solution, one deuterium atom incorporates into the dA of newly synthesized DNA, and is measured as the dA M+1 moiety. However, if using aqueous deuterated glucose ($^2H_2$-Glucose) as the DNA enrichment solution, two deuterium atoms incorporate into the dA of newly synthesized DNA, and the dA M+2 moiety should be measured. Other researchers in the field have utilized deuterated water with quantitation of the dA M+1

moiety[9,13] and aqueous deuterated glucose with quantitation of the dA M+2 moiety.[10–12] Therefore, a single methodology that is developed and optimized to concurrently measure dA and its stable isotopologues (e.g. dA M+1, dA M+2) would be advantageous.

The targeted component (2'-deoxyadenosine, $C_{10}H_{13}N_5O_3$, M.W. 251.24 Da) is a deoxynucleoside base found in genomic DNA. It forms hydrogen bonds with its complement DNA base pair, deoxythymidine (T). 2'-deoxyadenosine is a derivative of adenosine and differs by replacement of the (OH) group with (H) at the 2' position in the ribose moiety. Isotopologues are molecules that differ in isotopic composition. For example, hydrogen related isotopologues include water (H_2O, MW 18 Da), which can also exist as heavy water (^2H_2O, MW 20 Da) or tritiated water (^3H_2O, MW 22 Da). Water can also have an oxygen related isotopologue, such as doubly labeled water ($^2H_2{}^{18}O$, MW 22 Da). Although the GC does not normally separate isotopologues, a tuned mass spectrometer with unit resolution will separate isotopologues such as deoxyadenosine (dA or dA M0), dA M+1 (e.g. $^2H_1$), dA M+2 (e.g. $^2H_2$) and dA M+5 (e.g. $^{15}N_5$). One main advantage of using a triple quadrupole tandem MS is utilization of the multiple reaction monitoring (MRM) mode of analysis. The MRM mode significantly increases the detector signal-to-noise ratio, thus enhancing method sensitivity and reducing the amount of cells needed for DNA analysis.

Initial MS analysis on dA and its isotopologue derivatives utilized the electron impact (EI) mode of ionization. However, component fragmentation using EI mode resulted in a small molecular ion, which reduced method sensitivity. The use of chemical ionization in the positive ion mode (PCI) and methane reagent gas resulted in a softer ionization, larger abundance of dA molecular ion, and less fragmentation which significantly increased method sensitivity. To achieve an even softer ionization, isobutane was used as the PCI reagent gas, which provided the highest sensitivity for derivatives of dA and its isotopologues. In the PCI mode, the reagent gas is ionized in the ion source and "softly" transfers the charge to the component (e.g. dA). This results in a positive ion $[M+H]^+$ and increases the mass of the component by one Dalton. For example, the dA methylation derivative has a mass of 307 Da. Using MS in the PCI mode, dA, dA M+1, dA M+2, dA M +5 (I.S.) and 8-oxo-dA would have resulting masses of 308, 309, 310, 313, and 338 Da, respectively.

Additional GC-MS/MS optimizations were performed to increase method sensitivity for accurately detecting trace levels of deuterium enrichment into DNA of cells undergoing proliferation. GC optimizations included evaluating GC pulsed split-less head pressure, GC inlet temperature (°C), and inlet head pressure (psi). MS optimizations included evaluating PCI versus EI modes of ionization, type of PCI reagent gas (e.g. methane, isobutane). ion source temperature (°C), isobutane flow rate (ml/min), and quadrupole collision energy (eV). Optimized GC and MS set points were used in the final GC-MS/ MS method (Supporting Information, Figure 2).

For sample derivatization, the method utilized an on-line heated inlet gas phase technique to derivatize dA and its isotopologues. Briefly, the MethElute™ (trimethylanilinium hydroxide, pH ≥ 10) reagent formed methylcarbonium ions $[CH_3]^+$ in the heated GC injection port. Injected samples containing labile hydrogens (e.g. OH, NH) were rapidly methylated (CH_3) in less than 20 seconds (i.e. sample residence time in the GC heated injection port) (Supporting Information, Figure 3). As dA has four labile hydrogen's, the derivatization procedure added four methyl groups to the dA moiety. For dA, dA M+1, dA M+2, and dA M+5 (I.S.), the resulting methylated derivatives had masses of 307, 308, 309, and 312 Dalton (Da), respectively. The oxidative adduct 8-oxo-dA had 5 labile hydrogen's, resulting in a methylated derivative with mass of 337 Da.

Organic modifiers (solvents) were evaluated to determine their effect on MethElute™ derivatization of dA. As Fox *et al.*[10] used DMF and MethElute (1:1, v/v) in the final injection solution, DMF and other organic solvents were evaluated to determine their effects on dA derivatization. Both protic (hydrogen bond and acidic hydrogen) and aprotic organic solvents were evaluated as demonstrated in Supporting Information, Figure 4. In our experiments, addition of organic modifiers to the MethElute™ solution did not improve MRM response for the dA derivative or its isotopologues; therefore it only contributes to a dilution of the final sample, thereby decreasing method sensitivity.

The method employed the use of fast GC and low thermal mass (LTM) technology, which is a recent technology. Briefly, the LTM utilized a short column with narrow internal diameter and thin film thickness. Using a fast GC module with LTM technology, the analytical column can be rapidly and reproducibly heated at 120°C/min. The fast GC with LTM approach increased component sensitivity (i.e. increase peak height) and reduced analysis time, which provided high sample throughput capability. As seen in Supporting Information, Figure 5 and using the same analytical column dimensions, a GC oven ramp rate of 120°C/min reduced the retention time of dA by ~50%, and provided equivalent dA and dA M+1 calibration curves. When utilizing the fast GC capability of LTM technology, the analytical run time was quickly reduced to 7.5 min for method validation, with recent GC optimizations (e.g. increased flow rate) to further reduce the analysis time down to 4.5 min.

For work with potential future samples containing very small number of cells (e.g. 10,000 mouse cells) the current methodology was evaluated using dA at levels which would mimic complete recovery and hydrolysis of DNA from processing 10,000 cells. Therefore, standard curves using 3 μM dA with enrichments of 0.25–5.0% dA M+1, were evaluated on three analytical runs (i.e. 3 days). Back calculated results for the standard curves were comparable to standard curves generated using 20,000 cells. However, one potential limitation to preparing small numbers of mouse cells for analysis would be in assuring a high yield of DNA from cell lysing and extraction, and a high efficiency of DNA hydrolysis to its deoxynucleoside base pairs.

As other researchers have utilized $^{2}H_{2}$-glucose for their cell proliferation studies, we decided to add its MRM transitions to the current method. The stable isotope $^{2}H_{2}$-glucose can incorporate deuterium into the DNA of proliferating cells; however, it incorporates two deuterium atoms into dA, as opposed to one deuterium atom when using $^{2}H_{2}O$. The incorporation of two deuterium atoms will contribute to the dA M+2 isotopologue, which is detected using MRM transition m/z 310 → 164. Standard curves using 6 μM dA M0, 0.25–5.0% dA M+2, and 2 μM dA M+5 (I.S.) were evaluated over three days. The calibration curves were generated using a polynomial fit, which provided excellent back-calculated results and coefficient of determination. Supporting Information, Figure 6 displays MS MRM scans and calibration curves representing stable isotope infusion solutions using deuterated water or deuterated glucose. The only notable difference between the calibration curves is the y-intercept, which accounts for differences in natural isotope abundances of the dA M+1 and dA M+2 isotopologues.

Standards of 8-oxo-dA and 8-oxo-dG were prepared at 20 nM in methanol: MethElute™ (50:50, v/v) to determine method sensitivity for the DNA oxidative adducts. As sample preparation causing oxidative artifacts of dA and dG have been reported in the literature,[18,19] evaluations were made and artifacts (instrumental and/or process) were not detected at the nM level. However, 8-oxo-dG in MethElute™ was found to be unstable after several hours, and should not be evaluated using this derivatization technique.

Additional experiments were performed to allow for indirectly estimating the number of processed cells obtained from mouse experiments. This theoretical estimation would assume 100% DNA yield from cell lysing and hydrolysis, to the deoxynucleoside base pairs. Standards containing dA (0.1 to 6 μM) were prepared and evaluated, which theoretically approximates ~330 to ~19,778 processed mouse cells.[20,21] The GC-MS/MS method accurately quantitated levels as low as 0.1 μM dA, which theoretically represents processing several hundred mouse cells.

GC-MS/MS Fit-for-Purpose Method Validation

A fit-for-purpose method validation was performed for our studies on cGVHD. Validation parameters included linearity, accuracy, precision, lower limit of quantitation (LLOQ), limit of detection (LOD), selectivity, specificity, HLB extraction recovery and stability of the dA derivative. A brief description of each section of the method validation and corresponding validation results is discussed. Using component stock solutions, calibration standards containing a final concentration of 6 μM dA, enrichments of 0.25–20% dA M+1, and 2 μM dA M+5 (I.S.) were prepared using methanol and MethElute™ (1:1, v/v). For dA computations, method calibration was performed using Agilent MassHunter Quantitation software and component peak height. The calibration model used an average detector response factor from calibrators containing 6 μM dA and 2 μM dA M+5 (I.S.). The calibration standard contained 6 μM dA, as it corresponded to mouse sample preparations consisting of ~150 ng mouse genomic DNA (~19,000 cells). Using the 6 μM dA standard calibrator assumed having a high DNA yield and hydrolysis efficiency from the preparation of mouse cells.

For dA M+1 isotopologue computations, method calibration was performed using component peak height and Microsoft Excel 2010 software. Computations utilized a polynomial fit of X (i.e. dA M+1 %enrichment) versus Y (i.e. ratio of (dA M+1 / (dA M0 + dA M+1))). Excel calculated the polynomial fit and determined the calibration parameters and coefficient of determination (R^2). A typical calibration plot for dA M+1 %enrichment versus peak height ratio of dA M+1 and dA is portrayed in Supporting Information, Figure 5. For this method, the use of a polynomial fit (e.g. $y = -4E\text{-}05x^2 + 0.008x + 0.073$) provided consistent coefficient of determinations (R^2), and good back-calculated values for dA M+1 enrichment standards.

It should be noted that a non-zero positive y-intercept represents the isotopic background contribution from naturally occurring stable isotopes (e.g. ^2H, ^{13}C, ^{15}N), which will be found in the dA derivative. Processed samples that are either above or below the targeted 6 μM dA derivative can have different background isotopic contributions. For this reason, it was critical to process a known and targeted amount of mouse cells, and to quantitate using a matched dA calibration standard (e.g. ~19,000 processed mouse cells theoretically contains ~ 6 μM dA).

Instrument accuracy and precision was evaluated by intra and inter-day evaluations using prepared standards containing 6 μM dA, enrichments of 0.25–5.0% dA M+1, and 2 μM dA M+5 (I.S.). Intra-day (n=6) evaluation was performed by repetitive injection of the standards on the same day. Inter-day (n=6) evaluation was performed by injection of the standards on six different days. Accuracy is reported as % error (i.e. ((measured amount - nominal amount) / nominal amount)×100) and is determined by back calculation of each standard using the appropriate calibration model. For dA, the calibration model used average response factors, and for dA (M+1) the calibration model used a polynomial fit. Precision is reported as %C.V. (i.e. (standard deviation / mean) × 100). It is important to note that the instrument accuracy and precision results include the online heated GC inlet derivatization

of dA and its isotopologues. As seen in Table 1, the method demonstrated excellent instrument accuracy and precision for all calibration standards.

The lower limit of quantitation (LLOQ) and higher limit of quantitation (HLOQ) for the method is defined as the lowest and highest calibration standard, respectively, which can accurately and precisely be determined with less than 20% total error. For evaluating ~19,000 mouse cells, the LOQ for dA was 6 μM, as determined by replicate standards that were accurately and precisely determined. For dA (M+1), the LLOQ and HLOQ was 0.25% and 20%, respectively, with total errors of ≤ 20% (Table 1). The limit of detection (LOD) for the method was determined by dilution of calibration standards until the MS signal approached background noise (i.e. matrix and electronic). The method LOD was determined to be ~200 pM (~100 fg on-column) for derivatives of dA and its isotopologues. The MRM signal for each component at ~200 pM was greater than 3-times the background noise.

To address method specificity, component MRM transitions were determined as follows. An initial PCI MS full scan was used to verify the derivative component's molecular weight (i.e. MS1 precursor ion), followed by using the PCI MS product ion mode to determine a unique (specific) quantitation ion (i.e. MS3 detection ion). Optimal MRM transitions were used for each component and listed in Supporting Information, Table 3. The use of LTM capillary DB-17ms GC columns provided rapid and high resolution (selective) chromatography. Method column selectivity was consistently demonstrated by the absence of MS response from methanol: MethElute™ reagent injections, at the retention times of dA and its isotopologues. In addition, the MRM chromatograms were free of component carryovers from injections of mouse DNA extracts. Figure 1 demonstrates method selectivity and specificity for dA, dA isotopologues, and the 8-oxo-dA adduct.

A recovery study on dA and its isotopologues was performed using the 96 well plate Oasis® HLB solid phase extraction. Control samples were prepared in PBS and consisted of 6 μM dA, enrichment of 1.0% dA M+1, and 2 μM dA M+5 (I.S.). The control samples were extracted with the mouse DNA samples. The control samples had consistently high recoveries (>98%), and were compared against unextracted calibration standards.

A stability evaluation was performed to monitor the component's stability in MethElute™ reagent (pH ≥ 10). Standard solutions containing dA, the dA isotopologues and 8-oxodA were prepared in MethElute™ reagent, and were stored in autosampler vials at ambient lab temperature. These standards were compared to freshly prepared standards and evaluated over five days. All components were determined to be stable in the MethElute™ reagent for at least 5 days. The stability of mouse cell DNA extracts was at least 24 hrs., with longer evaluations not performed due to the small reconstitution volume (i.e. 15 μL) used for the GC-MS/MS analysis, and the potential loss of volume due to evaporation.

Method Limitations

The use of stable isotopic enrichment substances (e.g. $^{2}H_{2}O$), which incorporates into the DNA of proliferating cells, can present significant analytical challenges. Specifically, the challenge becomes quantitating trace levels of deuterium enrichment label (i.e. dA M+1) above natural stable isotopic background (e.g. ^{13}C, ^{15}N, ^{2}H). Since stable isotope enrichments for our studies can occur at trace levels (e.g. 0.25%), this analytical variability can become a significant contributor to total experimental error. Standards containing dA were prepared at increasing levels (e.g. 1, 20, 50 μM), and resulted in a non-linear increase in the natural stable isotopic background level of dA M+1. Therefore, for accurate measurement of the dA M+1 isotopic enrichment, it is critical to prepare dA standards at levels close to the expected amount of dA extracted from the mouse cells. For example, if

preparing 19,000 mouse cells for DNA analysis, 6 μM dA standards should be prepared using targeted enrichments of dA M+1.

There can be significant negative effects from residual water in the reconstituted sample used for GC-MS/MS analysis, as it can cause quenching of the MethElute™ derivatization. During the HLB extraction procedure, the elution step for dA and its isotopologues from the HLB sorbent uses 150 μL methanol. Although the methanol used was dried using molecular sieves, there is the potential for residual water from the buffer washes from the extraction procedure to be eluted with the sample. Even after vacuum evaporation is utilized to remove the elution solvent (methanol), it was important to visually ensure that the microvial containing the sample residue was completely dry. As demonstrated in Supporting Information, Figure 7, even 1% residual water can decrease (~17%) the MS signal of dA.

Commercial kits that were utilized for cell lysis (Promega Corporation) and DNA hydrolysis (Epigentek) have shown lot-to-lot variation in performance capability. For example, the Promega kits provided DNA yields from mouse cells ranging from 40–80%. This wide range can probably be attributed to the kits being developed for lysing cells and producing high purity DNA for other downstream assays (i.e. PCR). The Epigentek kits provided DNA hydrolysis efficiencies ranging from 60–90%. For all cell processing steps, it was important to avoid the use of EDTA and other metal chelators (e.g. EGTA). EDTA will bind to divalent cations (e.g. Ca^{2+}, Mg^{2+}), which are required by these enzymes as cofactors for DNA hydrolysis. As demonstrated in Supporting Information, Figure 8, even 500 μM EDTA can have significant negative effects (~30%) on DNA hydrolysis efficiency.

Method Application to Critical Translational Research

Deuterium stable isotope labeling was utilized in several mouse models of immunity, including control mice at immune homeostasis (n=2), mice undergoing syngeneic HSCT (n=3), and mice receiving AHSCT (n=7) with subsequent induction of cGVHD. Purified CD4+Foxp3+CD25+ (Tregs) and CD4+Foxp2- lymphocytes from spleens of each mouse cohort were then collected via FACS and characterized by flow cytometry. Flow cytometric analysis of spleen allowed us to quantify the total number of given cell type and its proportion within the organ. Concurrently with splenic T cell sorts, unsorted bone marrow cells were collected from each cohort and stored until subsequent processing culminating in GC-MS/MS analysis, with dA M+1 values used in cell division calculations of the respective spleen-derived T cells. The computation for determining percent fraction (f) new cells per day was the following: f = ((sample sorted splenic T cells (dA M+1) / sample bone marrow cells (dA M+1)) × 100), which maintains computational consistency to other researchers in the field[9].

FACS and GC-MS/MS analysis were performed on pooled cells from each subgroup. Cell division kinetics in AHSCT recipients indicated slower gain of splenic Tregs compared to CD4+ T non-Treg lymphocytes, while in syngeneic recipients these rates were similar, and in normal animals a reverse trend was observed (Figure 2). Evaluation of cell kinetics within distinct lymphoid compartments (lymph nodes, peripheral blood) and target organs of cGVHD (liver, thymus, skin) is necessary for generating a model of *in vivo* T cell behavior post AHSCT. This will identify mechanisms underlying cGVHD pathophysiology, and will become a powerful tool to investigate effects of therapeutic interventions on disease prevention and/or treatment in pre-clinical models. Furthermore, understanding of mouse *in vivo* T cell behavior (cell gain and loss from various T cell pools) provides the basis for mathematical modeling of T cell subpopulation dynamics in mice, and will subsequently allow interpretation of human peripheral blood lymphocyte kinetics measurements.

CONCLUSIONS

We have developed and validated a rapid GC-MS/MS method to quantitate deoxyadenosine and trace levels of its isotopologues (e.g. dA M+1) from limited mouse cell populations (e.g. Treg cells). In our studies, mice undergoing AHSCT received deuterated water starting on day of graft injection, allowing the DNA of proliferating cells to be labeled with deuterium, which was detectable in the dA M+1 isotopologue. Targeted and frequently limited cell populations (e.g. 20,000) were isolated and processed from the mouse lymphoid organs; with separation and purification using multi-parameter FACS.

The method employed readily available commercial kits to perform cell lysis, DNA isolation and hydrolysis to its deoxynucleoside base pairs. A simple solid phase extraction (HLB) was used to purify and concentrate the deoxynucleoside base pairs, followed by an on-line hot inlet gas phase sample derivatization technique, which took less than 20 seconds for completion. As our goal was to develop a sensitive high throughput method, fast chromatography was employed using low thermal mass (LTM) technology, and was coupled to a novel and recently marketed GC-MS/MS system. By incorporating the LTM technology and its narrow bore capillary column, the analytical run time was reduced from 21 min to 4.5 min. Method calibration curves using dA, dA M+1 enrichments of 0.25–20%, and dA M +5 (I.S.) provided quantitative results with excellent accuracy and precision. Additional capabilities of the GC-MS/MS method were developed to allow for quantitation of the dA M +2 isotopologue when using deuterated glucose to label the DNA, as well as the oxidative stress marker and DNA adduct, 8-oxo-deoxyadenosine (8-oxo-dA).

The high sensitivity and specificity of the method make it useful for investigating *in vivo* cell kinetics. The use of stable isotopic labeling in the form of deuterated water has been demonstrated safe to use in mouse and human studies, lending to its applicability in many fields of translational research. Although we describe the method in application to a specific field of translational cancer research, the approach can be utilized to elucidate mechanisms underlying a variety of disease conditions and disease-models that are immune-mediated, such as diabetes, HIV/AIDS, arthritis, inflammatory bowel disease, and multiple sclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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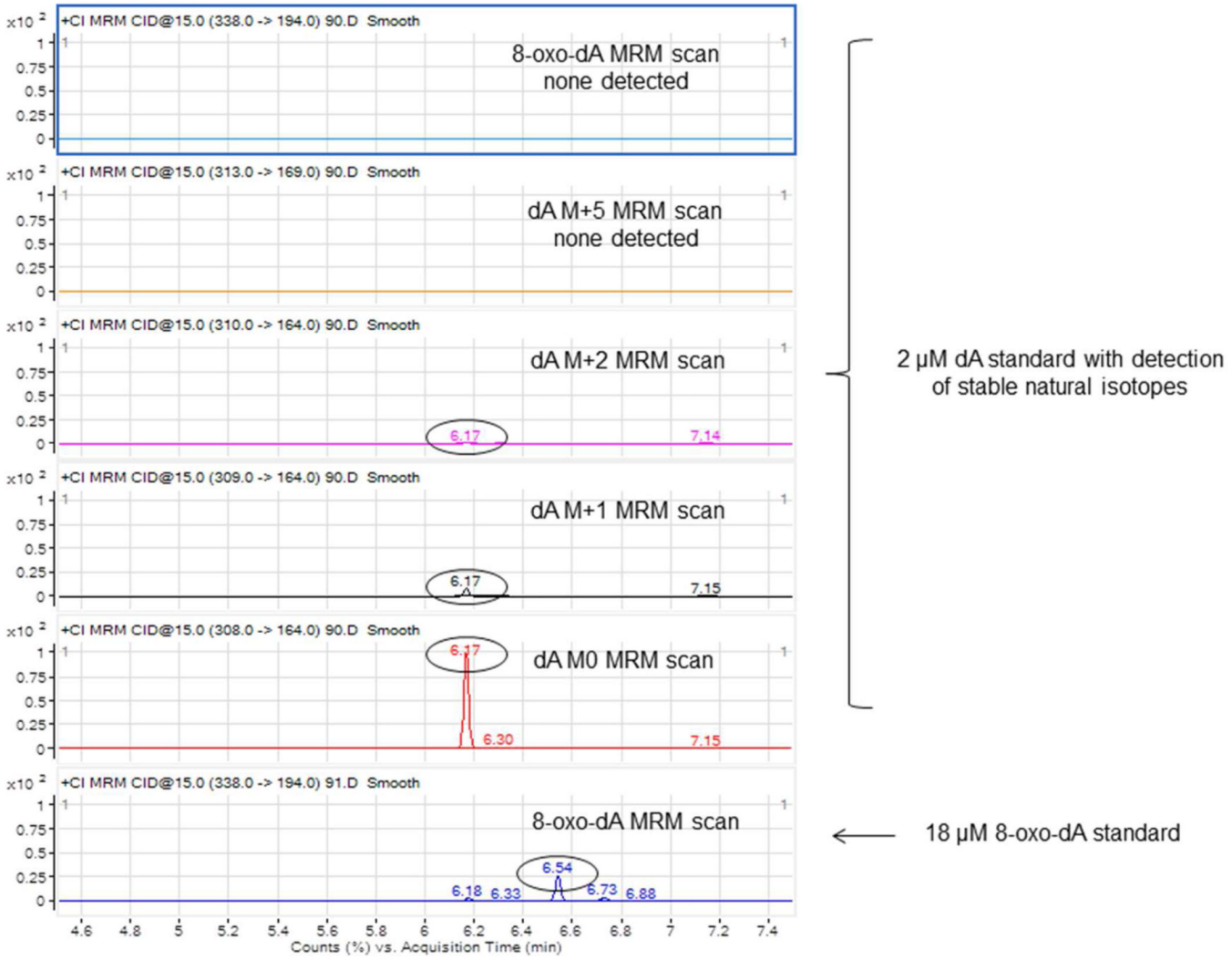

Figure 1.

Chromatogram overlays representing multiple reaction monitoring (MRM) on standards containing 2 μM dA and 18 μM 8-oxo-dA. The overlays from the 2 μM dA standard contain MRM scans that represent the consistent levels of stable natural isotopes found in 2 μM dA (i.e. dA M+1 (m/z 309→164), M+2 (m/z 310→164). The MRM scan for the 8-oxo-dA adduct in the 2 μM dA standard found no adduct detected, indicating a lack of detectable oxidation from the hot GC inlet derivatization procedure.

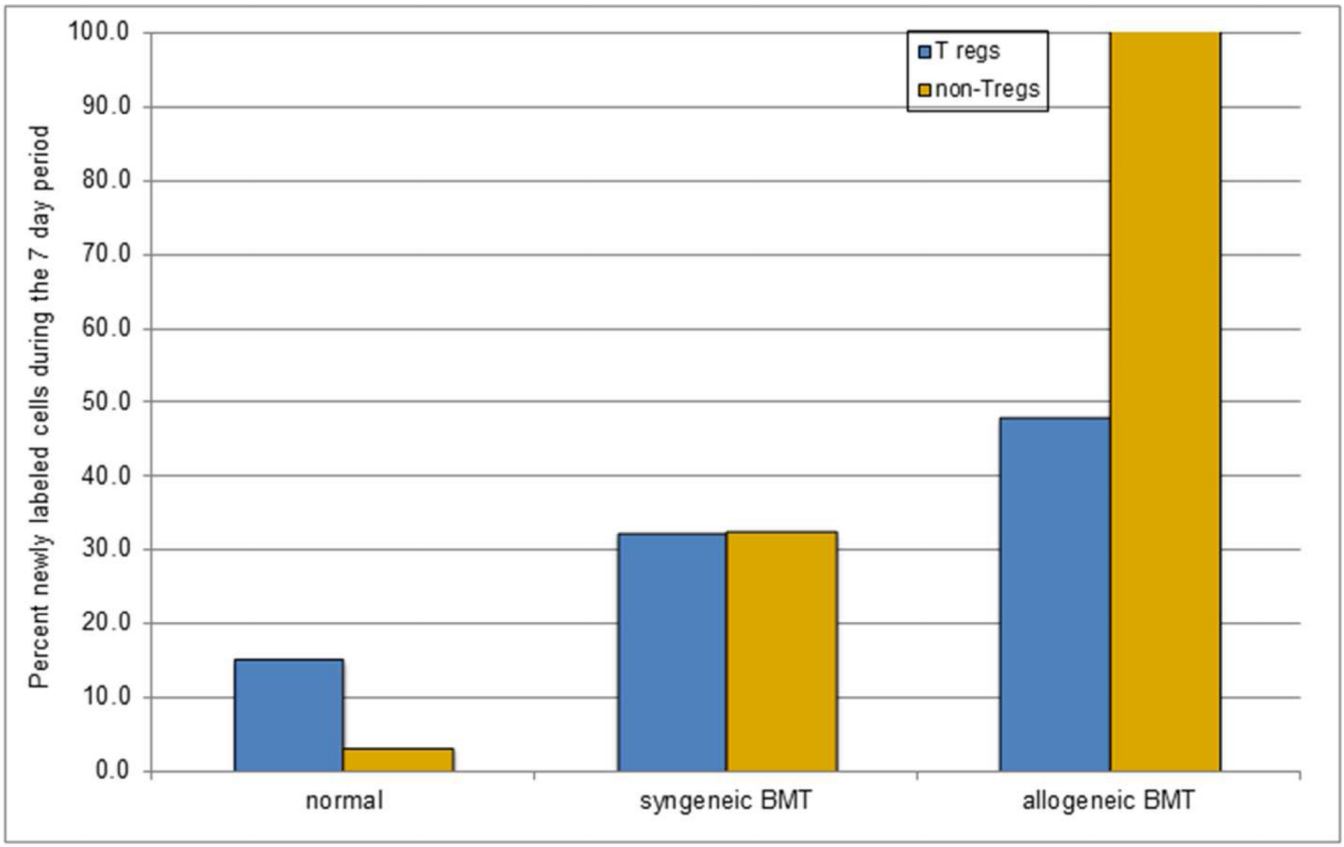

Figure 2.
Bar chart demonstrating labeling kinetics of pooled splenic T cells for days 28-35. Cellular gain within splenic CD4+ T cell subsets for normal mice, syngeneic and allogeneic BMT recipients, defined by percent newly labeled cells during a 7-day deuterium labeling period.

Table 1

Intra and Inter-day Accuracy and Precision Results for dA and Enrichments of dA M+1

Intro-day Accuracy and Precision (dA %M+1 enrichment)				
Nominal Conc.	**Measured Conc. (n=6)**	**Stdev.**	**% Error**	**% C.V.**
0.25	0.27	0.03	8.0	11.1
0.50	0.52	0.06	4.0	11.5
1.0	1.0	0.07	0.0	7.0
2.5	2.6	0.11	4.0	4.2
5.0	5.0	0.08	0.0	1.6
10.0	9.9	0.10	−1.0	1.0
20.0	19.5	0.50	−2.5	2.6
Inter-day Accuracy and Precision (dA %M+1 enrichment)				
Nominal Conc.	**Measured Conc. (n=6)**	**Stdev.**	**% Error**	**% C.V.**
0.25	0.28	0.02	12.0	7.1
0.50	0.51	0.06	2.0	11.8
1.0	1.0	0.06	0.0	6.0
2.5	2.5	0.08	0.0	3.2
5.0	4.9	0.05	−2.0	1.0
10.0	9.9	0.15	−1.0	1.5
20.0	19.0	0.73	−5.0	3.8
Intra and Inter-day Accuracy and Precision (6 μM dA)				
Nominal Conc.	**Measured Conc. (n=42)**	**Stdev.**	**% Error**	**% C.V.**
6.0	5.998	0.11	−0.04	1.9