Mutagenicity and Potential Carcinogenicity of Thiopurine Treatment in Patients with Inflammatory Bowel Disease

Truc Nguyen, Pamela M. Vacek, Patrick O’Neill, Richard B. Colletti, and Barry A. Finette

Abstract

The thiopurines azathioprine and 6-mercaptopurine (6-MP) are effective immune modulators and cytotoxic agents extensively used in the treatment of autoimmune diseases, graft rejection, and cancer. There is compelling epidemiologic evidence that thiopurine treatment increases the risk for a variety of tumors by mechanisms that are unclear. We investigated the in vivo mutagenicity of long-term thiopurine treatment by determining the frequency and spectra of somatic mutation events at the hypoxanthine phosphoribosyltransferase (HPRT) locus in peripheral T lymphocytes as well as the prevalence of mutant clonal proliferation in a cross-sectional analysis of data from 119 children and adults with inflammatory bowel disease (IBD). ANOVA and regression were performed to assess relationships among the frequency and spectra of HPRT mutations with disease, duration of illness, duration of treatment, and total therapeutic dose of azathioprine and 6-MP. We observed a significant increase in the frequency of somatic mutations in 56 subjects treated with thiopurines for IBD compared with 63 subjects not treated with thiopurines. This increase was related to both total dose ($P < 0.001$) and duration of treatment ($P < 0.001$). Comparative mutation spectra analysis of 1,020 mutant isolates revealed a significant increase in the proportion of all transitions ($P < 0.001$), particularly G:C to A:T transitions ($P < 0.001$). Combined analyses of two signatures for mutant clonality, HPRT mutation, and T-cell receptor $\beta$ CDR3 region unique gene sequence also showed a significant thiopurine-dependent increase in mutant cell clonal proliferation ($P < 0.001$). These findings provide in vivo evidence for mutation induction as a potential carcinogenic mechanism associated with chronic thiopurine intervention. [Cancer Res 2009;69(17):7004–12]

Introduction

Azathioprine and 6-mercaptopurine (6-MP) are effective immune modulators and cytotoxic agents used extensively for the treatment of autoimmune diseases (1), transplant graft rejection (2), and cancer (3). Their pharmacologic effects are complex and involve both inhibition of purine biosynthesis and incorporation of 2-deoxy-6-thioguanine nucleotides into DNA (4, 5). Cytotoxicity following thiopurine incorporation into DNA seems to require methylation of the thiol group, mispairing of S-methylthioguanine with thymine, with subsequent recognition and signaling by the mismatch repair (MMR) system (4).

A major continued concern is the broad-based epidemiologic evidence for duration (6) and dose-dependent (7–9) increases in the incidences of mesenchymal and solid tumors following thiopurine treatment (6–17). This associated cancer risk has resulted in azathioprine being classified as genotoxic and a human carcinogen (18, 19). Although the carcinogenicity of thiopurines has been attributed to their immunosuppressive effects, comparative epidemiologic studies with other immune modulators also suggest a thiopurine-specific contribution to cancer risk (9, 14, 15, 17). In particular, in vitro and animal investigations indicate that these agents are directly mutagenic (4, 20–25). In this regard, human biomonitoring investigations have observed a significant increase in the frequency of mutant T cells following thiopurine treatment for transplant rejection (26), systemic lupus erythematosus (27), and diabetes mellitus (28), but the etiology of these increases was not discerned. To date, the specific mechanism(s) responsible for the in vivo carcinogenicity of thiopurine treatment has not been elucidated.

In this study, we investigated potential in vivo carcinogenic mechanisms associated with thiopurine treatment by conducting a cross-sectional analysis of its mutagenicity by examining the frequency and spectra of somatic mutations at the hypoxanthine phosphoribosyltransferase (HPRT) locus in peripheral T cells as well as determining the prevalence of in vivo clonal expansion of mutant isolates in subjects with inflammatory bowel disease (IBD) in relation to disease type, duration of illness, duration of treatment, and total thiopurine dose.

Materials and Methods

Study population. The overall research design used is illustrated in Fig. 1. In vivo mutagenicity of thiopurine treatment was examined in 119 children and adults with either ulcerative colitis (UC) or Crohn’s disease (CD). Both of these chronic inflammatory bowel syndromes are associated with increased cancer risk at affected sites of inflammation (29) and lymphoma following thiopurine intervention (13). We elected to study the potential mutagenic effects of thiopurine treatment in subjects with IBD because only a portion of subjects receive thiopurine intervention, and in comparison with subjects with cancer or following transplantation, these subjects do not receive long-term treatment with other established mutagenic agents. Subjects with IBD were recruited from the pediatric and adult gastroenterology clinics at Fletcher Allen Health Care, the tertiary care facility at the University of Vermont, and consented with protocols approved by the Committee on Human Research. Diagnosis and therapeutic intervention were confirmed by participating gastroenterologists and a review of medical records. Heparinized blood samples were collected from 39 children ($\leq18$ y old) and 80 adults ($>18$ y old). Fifty-six subjects comprised a group of thiopurine-treated subjects who received azathioprine ($n = 40$), 6-MP ($n = 11$), or both ($n = 5$), including 19 children and 17 adults with CD and 7 children and 13 adults with UC (Table 1). Sixty-three subjects comprise a group of nonthiopurine-treated (nonthiopurine) subjects,
including 4 children and 13 adults with CD and 9 children and 37 adults with UC. All subjects previously or concurrently received other treatment modalities that included 5-aminosalicylic acid derivatives \((n = 98)\), corticosteroids \((n = 72)\), infliximab \((n = 21)\), metronidazole \((n = 30)\), and/or ciprofloxacin \((n = 7)\). The total equivalent dose (moles, converted to mmoles) of thiopurine exposure for subjects was determined by summing the total amount of thiopurines prescribed (grams) up to sample acquisition, divided by the molecular weight of each drug.

**HPRT T-cell cloning assay for determining somatic mutant frequency.** The HPRT T-cell cloning assay has been extensively used as a mutagenicity biomarker for determining the frequency and spectra of somatic mutation events in peripheral human T lymphocytes \((30, 31)\). Briefly, because HPRT encodes a phosphoribosylation enzyme in the purine salvage pathway, analysis of in vivo mutation events is accomplished by isolating HPRT-deficient T cells from peripheral blood through in vitro selection with 6-thioguanine (6-TG). This approach allows only cells with inactivating gene mutations to proliferate in the presence of 6-TG. The HPRT T-cell cloning assay has several additional characteristics that have made it a valuable human mutation biomarker system. HPRT mutant T cells arise in a normal T-cell milieu, uncomplicated by metabolic and genetic derangements inherent in diseased or malignant cells, allowing for the evaluation of mutagenic signatures related to exposure. Because the HPRT locus is located on the X-chromosome, a single mutation event can result in a selectable mutant.

![Figure 1.](image_url)
phenotype and is thus more sensitive than selectable autosomal gene loci. In addition, HPRT T-cell mutant isolates can be expanded in vitro and used for both mutation spectra and clonality analyses. The determination and statistical analysis for in vivo somatic HPRT mutant frequency (Mf) in peripheral T cells by cloning HPRT mutants following limiting dilution in the presence and absence of 6-TG have been previously described (32, 33).

**Molecular analysis of HPRT mutations and determination of mutant cell proliferation.** The HPRT biomarker system also allows for the analysis of mutation spectra changes associated with exogenous/iatrogenic exposures as well as the identification of independent in vivo sequential mutation events (34) and mutant clonal proliferations by simultaneously analyzing two independent clonality indicators: HPRT mutations and unique T-cell receptor β (TCRβ) CDR3 variable gene region sequences (35, 36). Experimental approaches, methods and interpretations of mutation spectra, and comparison of HPRT mutations and TCRβ CDR3 variable gene regions of mutant isolates for determining mutant proliferation have been previously described (34–36). HPRT mutation spectra were determined by analyzing up to 10 mutants per thiopurine-treated subject and up to 5 mutants per nonthiopurine-treated subject. HPRT mutations were characterized by performing reverse transcription-PCR (RT-PCR) amplification from $-1 \times 10^3$ cells to identify mutations within coding regions, exon exclusions, or intron inclusions. Single exon or multiplex genomic PCR of HPRT gene regions (37) was performed to identify splice mutations, deletions, or insertions. HPRT mutants that showed exclusion of exons 2 to 3 in cDNA were screened for V(D)J recombinase-mediated deletions of HPRT exons 2 to 3 with specific primers spanning that region (38). PCR products were either separated by gel electrophoresis, extracted, and purified via Qiagen QIAquick gel extraction kit or treated with 3.75 units of exonuclease I and 1.25 units of shrimp alkaline phosphatase before DNA sequencing.

The characterization of the TCRβ CDR3 variable gene regions and HPRT mutations in mutant isolates provides two independent measures for clonal proliferation that can be followed longitudinally (Supplementary Fig. S1; refs. 34, 35). For TCRβ variable gene sequence analysis, RT-PCR–generated cDNA was amplified using a primer to the TCRβ constant region and a mix of 26 TCRβ V region primers (39). The PCR products were then processed and sequenced as described above. The prevalence of in vivo clonal expansion was determined by analyzing 621 HPRT mutations and 611 TCRβ CDR3 variable gene region sequences from 114 subjects from whom two or more mutants were available (Supplementary Fig. S1; Supplementary Table S2). The in vivo frequency of independent mutation events (MutFreq) was ascertained by multiplying each subject’s HPRT Mf by the ratio of independent mutation events to the total analyzed mutants.

**Statistical analysis.** Because HPRT Mf has a log-normal distribution, all statistical analyses were based on its natural logarithm (lnMf), which is related to age and nonselected cloning efficiency (CE; ref. 32). We therefore adjusted for age and CE by including them as covariates in all analyses of lnMf (40). A summary of each subject’s gender, age, duration of illness, type and treatment duration, total dose of thiopurine, CE, Mf, lnMf, and MutFreq is provided (Supplementary Table S1). Analysis of covariance was used to compare differences in lnMf among controls, nonthiopurine- and thiopurine-treated subjects, as well as between children and adults with UC and CD. The Student-Newman-Keuls test was used to adjust for multiple pairwise comparisons. In thiopurine-treated subjects, the effects of treatment duration and total dose on Mf and MutFreq were assessed by regression analyses. A $\chi^2$ test was used to compare the percents of thiopurine- and nonthiopurine-treated subjects with mutant cell proliferation, and a Mantel-Haenszel test for linear association was used to assess the dose dependence of increases in the percent of subjects with mutant cell expansions. Mutation spectra data were analyzed using logistic regression to assess the statistical significance of differences in the proportions of specific mutations among controls (Supplementary Table S3) and nonthiopurine- and thiopurine-treated subjects. Subjects under the age of 12 were excluded from mutation spectra analyses because differences in the age distributions of normal controls and IBD subjects preclude adjustment for age-related spectra changes in this group. Age group (12–18 or ≥19 y) and sex were included in the regression models as covariates. Although mutations from the same subjects may be correlated, we could not use a hierarchical regression model in these analyses because the spectra data for adult controls did not contain unique subject identifiers. The effects of treatment were determined by comparing data only from the thiopurine- and nonthiopurine-treated subjects. Hierarchical logistic regression models with age group (12–18 or ≥19 y), thiopurine treatment (yes or no), and treatment by age group interaction were fitted to

### Table 1. Duration of illness, total dose, and frequency of HPRT T-cell mutants in subjects with IBD

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Number</th>
<th>Age (y)*</th>
<th>Duration of illness (y)*</th>
<th>Total thiopurine dose (mmol)*</th>
<th>Mf $\times 10^{-6}$*</th>
<th>lnMf $\times 10^{-6}$*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal controls</td>
<td>280</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Children</td>
<td>49</td>
<td>5.7 (4.6)</td>
<td>13.7 (9.0)</td>
<td>2.3 (2.2)</td>
<td>0.4 (1.0)</td>
<td>2.4 (0.7)</td>
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<tr>
<td>Adults</td>
<td>231</td>
<td>52.8 (16.0)</td>
<td></td>
<td>137 (9.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No thiopurine treatment</td>
<td>46</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UC</td>
<td>37</td>
<td>39.7 (15.9)</td>
<td>7.8 (8.2)</td>
<td>0.0</td>
<td>23.7 (38.3)</td>
<td>2.5 (1.1)</td>
</tr>
<tr>
<td>Adults</td>
<td>17</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>UC</td>
<td>4</td>
<td>15.8 (12)</td>
<td>1.2 (2.0)</td>
<td>0</td>
<td>3.9 (1.4)</td>
<td>1.3 (0.4)</td>
</tr>
<tr>
<td>Adults</td>
<td>13</td>
<td>42.9 (10.4)</td>
<td>11.2 (9.3)</td>
<td>0</td>
<td>126 (7.3)</td>
<td>2.4 (0.5)</td>
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<tr>
<td>UC</td>
<td>20</td>
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<tr>
<td>Adults</td>
<td>7</td>
<td>14.9 (4.4)</td>
<td>4.1 (3.1)</td>
<td>458.1 (354.9)</td>
<td>6,340.4 (12,287)</td>
<td>5.5 (3.7)</td>
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<tr>
<td>Adults</td>
<td>13</td>
<td>30.7 (11.1)</td>
<td>4.3 (3.5)</td>
<td>459.7 (697.8)</td>
<td>6,246.6 (20,612.6)</td>
<td>4.0 (3.3)</td>
</tr>
<tr>
<td>CD</td>
<td>36</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adults</td>
<td>19</td>
<td>15.0 (3.3)</td>
<td>3.9 (3.6)</td>
<td>264.4 (314.7)</td>
<td>368.2 (617.2)</td>
<td>4.1 (2.1)</td>
</tr>
<tr>
<td>Adults</td>
<td>17</td>
<td>33.6 (13.4)</td>
<td>9.4 (7.9)</td>
<td>501.2 (348.7)</td>
<td>15,768.2 (39,664.8)</td>
<td>6.0 (3.6)</td>
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</table>

*Mean (SD).

† Mf and lnMf data summarized here have not been adjusted for CE and age. Mean lnMf is computed from the individual log-transformed Mf value.
Figure 2. Comparative analysis of adjusted HPRT Mf with respect to duration of illness, duration of treatment, and total dose of thiopurines. Mf values have been adjusted for the effects of age and CE by normalizing to the average age and CE for each subject (35). A, relationships between adjusted Mf and duration of illness in nonthiopurine- and thiopurine-treated subjects. B, relationships between adjusted Mf and duration of thiopurine treatment in IBD subjects as well as the relationships between adjusted Mf and treatment duration of different thiopurine interventions. C, relationships between adjusted Mf and MutFreq and total thiopurine dose in IBD subjects.

determine if the effect of treatment differed in adults and children. Similar models were fitted using thiopurine treatment categories based on the total dose (0, 1–133, 134–289, 290–727, and ≥728 mmol) to determine if the proportion of a particular class of mutation displayed a dose-response relationship. The nonzero categories correspond to dose quartiles for all thiopurine-treated subjects. All of these models included study subject as a random effect to account for potential correlation between mutations from the same individual.
Results

In vivo HPRT Mf in subjects with IBD. Unadjusted HPRT Mf and lnMf data with respect to type of IBD and thiopurine treatment for the 119 children and adults studied as well as previously reported data used for controls (32, 40, 41) are summarized in Table 1. The effects of IBD and nonthiopurine treatment on the in vivo background HPRT Mf were initially assessed by comparing lnMf in nonthiopurine-treated subjects with controls after adjustment for age and CE. A statistically higher lnMf was observed in one subgroup, nonthiopurine-treated children with UC (P < 0.05), whereas lnMf in all the other nonthiopurine-treated IBD groups was not significantly elevated. Analysis of lnMf for the nonthiopurine-treated IBD group as a whole showed no statistical difference from controls, indicating that IBD itself and nonthiopurine interventions did not significantly affect the background HPRT Mf in nonthiopurine-treated IBD subjects.

For the 56 thiopurine-treated subjects, total thiopurine dose ranged between 3.8 and 2,379 mmol taken over 0.04 to 14.1 years. We observed a significantly higher mean lnMf in thiopurine-treated subjects compared with controls and nonthiopurine-treated subjects (P < 0.05; Table 1). Importantly, our subanalysis revealed no significant difference in lnMf between thiopurine-treated IBD children or adults or between thiopurine-treated IBD subjects with UC or CD. Regression analysis revealed no significant increases in lnMf associated with duration of illness for nonthiopurine-treated subjects (Fig. 2A; Supplementary Table S1). In contrast, we observed a significant increase in lnMf in thiopurine-treated subjects associated with duration of illness (P = 0.004; Fig. 2A), but of importance, this association was not significant after adjustment for either duration of treatment or total thiopurine dose. Specifically, the significant increases in lnMf in thiopurine-treated subjects were related to both duration of treatment (P < 0.001; Fig. 2B; Supplementary Table S1) and total thiopurine dose (P < 0.001; Fig. 2C) independent of duration of illness. The estimated rate of increase in lnMf in thiopurine-treated subjects was 0.776 per year of treatment, corresponding to a doubling in Mf. The estimated increase in lnMf with each 100 mmol increase in total dose was 0.467, corresponding to a 67% increase in Mf. The rates of increase in lnMf in thiopurine-treated subjects did not differ significantly between treated children and adults or treated UC and CD subjects. The rates of increase were also not drug specific because there were no significant differences in lnMf among subjects treated with only azathioprine, 6-MP, or both agents (Fig. 2B; Supplementary Table S1).

Prevalence of in vivo mutant cell clonal proliferation and frequency of independent somatic HPRT mutations (MutFreq). To gain insight into the potential effects of in vivo mutant clonal expansion and selection for HPRT mutants on the treatment-specific increases in mutant frequencies (MF), we investigated the relationship between the prevalence of mutant clonal proliferation and the calculated MutFreq in thiopurine- and nonthiopurine-treated IBD subjects.

We determined whether thiopurine treatment results in an increase in the prevalence of clonal proliferation of HPRT mutants by comparing 621 characterized HPRT mutations and 611 TCRβ regions from 876 HPRT mutants from 114 nonthiopurine- and thiopurine-treated IBD subjects (Supplementary Table S2; Supplementary Fig. S1). The percents of nonthiopurine-treated children and adults in which there were two or more HPRT mutant isolates associated with mutant clonal expansions were 0% and 14.6%, respectively (Fig. 3A). In contrast, there were significant increases in the percents of thiopurine-treated children (72.2%) and adults (63.3%) with mutant clonal expansions compared with nonthiopurine-treated subjects (P < 0.001; Fig. 3A). There were also significant dose-dependent increases in the proportion of subjects with mutant clonal expansions (P < 0.001; Fig. 3B).

The prevalence of in vivo mutant clonal expansion in these subjects was then used to correct for the possible contribution of in vivo mutant proliferation on the treatment-specific increases in lnMf to estimate the frequency of independent in vivo somatic mutation events (MutFreq). For thiopurine-treated subjects, the MutFreq was significantly lower than Mf (P < 0.001) as a consequence of mutant clonal expansions associated with treatment. But of importance, we also observed significant increases in MutFreq in thiopurine-treated subjects as seen with Mf that correlated specifically with treatment duration and total dose (P < 0.001; Fig. 2C; Supplementary Table S1). This strongly suggests that the significant increases in MutFreq following thiopurine treatment are not the result of selective in vivo expansion of preexisting HPRT mutants.

Mutation spectra analysis. The in vivo mutagenicity of thiopurine treatment was further examined by comparing 648
independent mutations from a composite of controls and 372 independent mutations from 105 IBD subjects 12 years and older (Supplementary Tables S2 and S3). Nineteen percent of the randomly selected HPRT mutants from IBD subjects remained uncharacterized, which is consistent with previous human biomonitoring HPRT spectra studies (36, 42). Reasons for the uncharacterized mutants include (a) exon exclusions in mutants from female subjects in which splice alterations and deletions could not be confirmed or detected; (b) no cDNA detected, with genomic PCR products of exons 1 and 9 showing no mutations; (c) cDNA revealed no mutation; and (d) incomplete analysis.

We observed no significant differences in the spectra of mutations between nonthiopurine-treated subjects compared with controls (Fig. 4A; Supplementary Tables S2 and S3). This establishes that there is no detectable IBD disease or non-thiopurine treatment–specific differences in the proportions of mutations. In contrast, there was a significant increase in the proportion of all transitions in thiopurine-treated subjects (52.9%) compared with nonthiopurine-treated subjects (39.8%; P = 0.016) and controls (34.4%; P < 0.001) after adjustment for age and multiple observations per subject. In particular, there was a significant increase in the proportion of G:C to A:T transitions at non-CpG dinucleotides in thiopurine-treated subjects (38.3%) compared with nonthiopurine-treated subjects (27.7%; P = 0.037) and controls (20.7%; P < 0.001; Fig. 4A; Supplementary Tables S2 and S3). The increase in transitions was also accompanied by a

Figure 4. Mutation spectra in nonthiopurine- and thiopurine-treated IBD subjects (≥12 y) and normal controls. A, mutation spectra distribution in nonthiopurine- and thiopurine-treated children and adults combined and separate compared with normal controls. The “other classes” of mutations include exon deletions, microdeletions, nucleotide duplications or losses, insertions, and complex insertion/deletion mutations. B, relationship between the proportion of G:C to A:T transitions at non-CpG dinucleotides and total dose of thiopurine treatment.
concomitant significant decrease in the proportion of all transversions among thiopurine-treated subjects (22.8%) compared with controls (34.6%; \( P = 0.021 \)).

We also observed a significant dose-dependent increase in the proportion of all transitions \( (P = 0.013) \), particularly G:C to A:T transitions \( (P = 0.045) \) at non-CpG dinucleotides after adjustment for age and multiple observations per subject, which supports our overall spectra findings (Fig. 5; Supplementary Table S2). This increase in G:C to A:T transitions is consistent with the in vitro mutagenic signature observed for these drugs (4, 20, 22).

Thiopurine treatment also resulted in a distinct distribution of unique G:C to A:T transitions at non-CpG dinucleotides at both coding and noncoding/splice site base pair positions in thiopurine-treated subjects compared with nonthiopurine-treated subjects (Fig. 5; Supplementary Table S4). There was an increase in the occurrence of unique G:C to A:T transitions at non-CpG dinucleotides \( (n = 38, \text{ compared with } n = 32) \) at both coding and noncoding sites and in the number of unique sites \( (n = 18, \text{ compared with } n = 12) \) in thiopurine-treated subjects compared with nonthiopurine-treated subjects. There were 20 common sites (coding and noncoding) in which these transitions occurred in both nonthiopurine- and thiopurine-treated subjects. The most frequent mutation coding sites (more than three events) for G:C to A:T transitions at non-CpG dinucleotides in nonthiopurine-treated subjects were at positions 538, 551, and 617. For thiopurine subjects, the most frequent coding sites were at positions 3, 197, 463, 485, 539, and 617. Of interest is that G:C to A:T transitions at non-CpG dinucleotides at positions 463 and 539 in thiopurine-treated subjects were not observed in nonthiopurine-treated subjects. Analogous findings were also observed at splice site sequences. In addition, the distribution of these transitions at coding and noncoding regions is consistent in each treatment group and distribution subcategory. These data suggest that the distribution of G:C to A:T transitions at non-CpG dinucleotides in thiopurine subjects represents both thiopurine-specific and preexisting mutations and not simply an increase in spontaneous or hotspot mutation events.

**Discussion**

Investigations attempting to link treatment-specific mutations to carcinogenic risk in humans are extraordinarily difficult because of the necessity to control for a multitude of complex variables, including the genetic and cellular effects of the underlying
disease(s), duration and total dose of each therapeutic intervention, as well as other environmental exposures. Furthermore, tumors themselves are genomically unstable, making potential primary and secondary cancer-relevant mutations difficult to discern. With respect to this study, definitively linking thiopurine mutation induction in IBD subjects to the formation of specific tumors is currently not possible because data for cancer-specific mutations in tumors from subjects who only received thiopurine treatment does not exist. Hence, identifying the in vivo induction of treatment-specific mutations in nontransformed cells is an extremely important approach for elucidating potential carcinogenic mutagenic events directly in subjects receiving this treatment.

In this study, we provide evidence that chronic thiopurine treatment results in a dose-dependent increase in the in vivo frequency of somatic mutations, particularly a treatment-specific increase in G:C to A:T transitions at non-CpG dinucleotides, which is consistent with the in vitro mutation signature of these drugs (4, 22). These data strongly suggest that thiopurine intervention is mutagenic in humans. These findings were ascertained by determining the frequency and spectra of somatic mutations at the HPRT locus in peripheral human T cells and an examination of in vivo preexisting or induced mutant cell clonal proliferation by combining the analysis of two distinct clonality signatures (35). Because the mutation events observed were investigated in subjects receiving chronic thiopurine treatment, the possibility exists for the in vivo selection and enrichment of preexisting HPRT mutants with potential growth advantages that may influence the relative contribution of thiopurine therapy on the observed Mf. This effect is unlikely because we observed a significant dose-dependent increase in MutFreq, which has been corrected for both preexisting and treatment-induced clonal expansion of mutant cells. The potential in vivo cytotoxic effects of thiopurines on proliferating non-HPRT mutants with the concomitant survival of HPRT mutants could also theoretically have influenced our results. This seems unlikely as well because we concurrently observed significant dose-dependent mutation spectra changes. The latter observations establish the in vivo mutagenicity of thiopurine intervention because significant spectra changes would not occur if this treatment simply enriched for the expansion of preexisting HPRT mutants. It is also important to note that all of these findings were independent of the underlying disease, duration of disease, and other therapeutic interventions.

We were unable to specifically evaluate the effects of other potential environmental exposures because of limited data. The most important exogenous exposure likely to influence our observations would be environmental tobacco smoke, which has previously been shown to result in an increase in V(D)J recombinase-mediated deletions in newborns (43) and no spectra changes or an increase in the proportion of G:C to T:A transversions in adults (44, 45). This is in contrast to the significant proportional decrease in transversions and an increase in G:C to A:T transitions at non-CpG dinucleotides observed in thiopurine-treated subjects.

The induction of G:C to A:T transition mutations by thiopurine treatment is likely the result of incorporation of 6-TG nucleotides into DNA and subsequent mispairing with thymine in replicating cells (4, 20, 22, 25). Support for the carcinogenicity of a thiopurine-specific mutagenic mechanism can be inferred from epidemiologic evidence correlating azathioprine treatment with significantly higher incidences of solid tumors, lymphoproliferative disorders, and skin cancers when compared with other immune modulators, including mycophenolate mofetil, which closely resembles thiopurines in its mechanism of action (9, 14, 15, 17, 25, 46). Mycophenolate mofetil primarily inhibits IMP dehydrogenase, resulting in the inhibition of purine synthesis, whereas azathioprine and 6-MP exert their cytotoxicity by inhibiting purine synthesis and by incorporation into DNA. This suggests that the increased cancer risk associated with thiopurine treatment is associated with the mutagenicity of these agents following DNA incorporation.

The significant increase in the prevalence of treatment-specific cell proliferation may represent an additional mechanism for accumulating G:T mismatches from unrepaird replication errors (47) as well as by enhancing incorporation of 6-TG nucleotides during DNA replication. Clonal proliferation among mutants, with the associated failure to find similar patterns among nonmutant cells, further implicates cell proliferation as a significant in vivo mutagenic mechanism.

Cellular resistance to thiopurines has been shown in vitro to select for cells defective in MMR, whereas microsatellite instability has been observed in acute myelogenous leukemia from transplant patients following azathioprine treatment (8). It is not known whether our findings could also be attributed to the in vivo selection of MMR-defective cells and the in vivo emergence of cells with genomic instability.

Our data indicate that thiopurine treatment could potentially lead to somatic mutations at disease-specific loci in actively replicating tissue cell populations, including (a) the bone marrow, which has been shown to incorporate higher levels of 6-TG nucleotides (48), and (b) activated immune cells and epithelial tissues undergoing repeated cycles of repair and cell regeneration, especially in subjects with IBD and chronic graft-versus-host disease (29, 49). Lymphocytes and skin epithelium of patients who received thiopurine treatment have also been shown to incorporate 6-TG nucleotides (24, 50). In this study, we also observed an increase in G:C to A:T transitions at non-CpG dinucleotides in clonally proliferating activated T cells, some of which may have a recent precursor origin (data not shown). Altogether, these observations provide an important connection between the mutagenicity of thiopurine intervention in these cell populations and the increased risk of leukemia (8), lymphoma (7, 12, 13), and skin cancer (9, 10, 17).

In this cross-sectional study, we provide evidence that suggests that thiopurine intervention is mutagenic, resulting in a dose-dependent increase in the frequency of somatic mutations and treatment-specific mutation induction of G:C to A:T transitions at non-CpG dinucleotides. These findings provide direct in vivo evidence for mutation induction as a potential carcinogenic mechanism associated with chronic thiopurine intervention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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