Objective: To prospectively determine optimal levels of 6-thioguanine nucleotide for disease remission in patients with immunobullous disease treated with azathioprine.

Design: Prospective, longitudinal study. Laboratory tests and clinical evaluations were performed monthly for 6 months and then every 2 to 3 months (median follow-up, 13.4 months).

Setting: Tertiary care medical center.

Patients: Twenty-seven patients with immunobullous disease treated with azathioprine were enrolled during a 2-year period. Twelve met the criteria for evaluation of optimal levels of 6-thioguanine nucleotide.

Main Outcome Measures: Blood levels of 6-thioguanine nucleotide, 6-methylmercaptopurine, and thiopurine methyltransferase by polymerase chain reaction and enzyme activity were measured longitudinally during treatment.

Results: The range of 6-thioguanine nucleotide was 48 to 457 pmol/10^8 red blood cells (RBCs), with an average optimal level of 190.7 pmol/10^8 RBCs for all patients. The mean optimal levels were 179.4 and 205.6 pmol/10^8 RBCs for pemphigus and pemphigoid, respectively. Limited disease required less 6-thioguanine, with a mean of 145.3 pmol/10^8 RBCs. Longitudinal induction of thiopurine methyltransferase activity was observed during treatment. Patients with recalcitrant disease showed higher induction of enzyme activity (with an increase of 9.1 to 23.6 U/mL of RBCs above baseline) than did those with responsive disease.

Conclusions: Optimal levels of 6-thioguanine nucleotide metabolites for disease remission in dermatology patients are 150 to 300 pmol/10^8 RBCs. High levels of the inactive metabolite 6-methylmercaptopurine and induction of thiopurine methyltransferase are associated with recalcitrant disease.


AZATHIOPRINE WAS APPROVED BY THE US FOOD AND DRUG ADMINISTRATION IN THE 1960s FOR IMMUNOSUPPRESSION AFTER SOLID ORGAN TRANSPLANT. IT HAS BEEN USED BY DERMATOLOGISTS AS A CORTICOSTEROID-SAVING AGENT FOR THE PAST 30 YEARS. THE MECHANISM OF ACTION OF AZATHIOPRINE IS NOT CLEAR, BUT IT IS THOUGHT TO INVOLVE THE ACTIVE CYTOTOXIC 6-thioguanine nucleotides (6-TGNs).

These metabolites were initially thought to exert their antiproliferative action on lymphocytes through incorporation into DNA. More recently, they have been shown to inhibit the CD28-dependent Rac1 activation of CD4+ T lymphocytes.

As with most prodrugs, the pharmacokinetics of azathioprine have been difficult to study. Azathioprine is metabolized by the liver to 6-mercaptopurine, which in turn is converted to either the active and cytotoxic 6-TGN or the inactive 6-methylmercaptopurine (6-MMP). Because measurement of the azathioprine drug level has had limited therapeutic value, the standard of care for monitoring patients treated with azathioprine has been to perform frequent complete blood cell counts and liver function tests in addition to measuring thiopurine methyltransferase (TPMT) activity 1 time before treatment, as proposed in 1995 by Snow and Gibson. An enzyme with previously described genetic polymorphisms, TPMT is not the only enzyme responsible for the metabolism of the cytotoxic 6-TGN, but it is the main one.
involved in the well-documented idiosyncratic hematopoietic toxic effect of azathioprine. Studies of inflammatory bowel disease and renal transplants have evaluated 6-TGN metabolite levels in red blood cells (RBCs) as a measure of therapeutic drug levels of azathioprine. The 6-TGN target level for patients with inflammatory bowel disease was reported as 235 pmol/8 × 10^8 RBCs or higher. In patients who have undergone renal transplant, a 6-TGN level of 100 to 200 pmol/8 × 10^8 RBCs was suggested as optimal to reduce the incidence of rejection and to avoid leukopenia. Similar criteria have not been determined for dermatologic diseases. As seen from the previous studies, different levels of 6-TGN are required for different diseases, indicating that the 6-TGN level required for disease remission may be disease specific rather than drug specific.

We present our findings from prospective monitoring of therapeutic levels of 6-TGN in the RBCs of patients treated with azathioprine for immunobullous disease. Our primary objective was to determine an optimal therapeutic level of 6-TGN for disease remission. Our secondary objective was to measure longitudinally the level of the inactive metabolite 6-MMP and the enzyme activity of TPMT to assess their roles in monitoring patients receiving azathioprine.

**METHODS**

A prospective study was conducted of dermatology patients who received azathioprine for immunobullous disease as recommended by their treating physician. The protocol design and enrollment criteria were approved and monitored by our institutional review board. Signed informed consent was obtained from each patient before enrollment in the study. Monthly blood samples were collected for complete blood cell counts, indirect immunofluorescence titers of circulating antibodies, and liver function tests. In addition, an extra 10-mL vial of whole blood was collected for measurement of 6-TGN, TPMT, and 6-MMP.

The TPMT was assayed in RBC lysates with 6-mercaptopurine as the methyl acceptor and 5-adenosymethionine as the methyl donor. After a 1-hour reaction period, the methylated product of TPMT (6-MMP) was quantitated by mass spectrometry with an internal standard of tritium-labeled 6-MMP (2H1-6-MMP). Thioguanine metabolites were measured in whole blood generally as previously described. Sample treatment involved perchloric acid deproteinization with dithiothreitol and hydrolysis of the thiopurine nucleotides to free bases by heating of the acid extract. The hydrolysate was subjected to reverse-phase high-performance liquid chromatography for separation and quantitation of 6-TGN and the 6-MPP hydrolysis product. The TPMT genotype was determined by sequencing exons 7 and 10 of TPMT in 20 samples (3730 DNA Analyzer; Applied Biosystems, Foster City, Calif). Each 20-ng sample was amplified with polymerase chain reaction (PCR) and the following primers: exon 7 forward primer, 5'-CTCCACACCCAGGTCCACACATT-3'; exon 7 reverse primer, 5'-GTATAGTATCTAAAAATTAGACACCTAAAC-3'; exon 10 forward primer, 5'-AATCCCTGATGTCATTCTTCATAG-3'; and exon 10 reverse primer, 5'-ACATCATATACTCCTCC-3'.

Patients initially received a high prednisone dose, and treatment with azathioprine began either at the same time or shortly thereafter. The azathioprine dose was determined on the basis of the initial measurement of TPMT. Heterozygous patients with high levels of TPMT (>13.7 U/mL of RBCs) were treated with standard doses of azathioprine (up to 250 mg/d). Heterozygous patients with TPMT levels from 5 to 13.7 U/mL of RBCs were treated with doses of azathioprine of 25 to 75 mg/d. We did not enroll any patients who were TPMT deficient. The prednisone dose was tapered when the disease was suppressed, and no new blisters were observed. Flares during the prednisone taper were treated with topical corticosteroids if minor or with an increase in azathioprine until the maximum dose was reached. At monthly intervals for the first 6 months of the study, patients were evaluated for clinical response, which included evaluation of disease severity on a 4-point scale (0, unchanged or worsened; 1, mild improvement of 25%-74%; 2, moderate improvement of 75%-99%; and 3, no residual or new blisters and disease in remission); the adverse effect profile, including objective and subjective aspects; laboratory test values; concomitant medications; and the current dose of azathioprine and prednisone. After the first 6 months, the clinical evaluation and laboratory tests were performed every 2 to 3 months. The optimal level of 6-TGN required for remission was considered to be the level of 6-TGN when the daily prednisone dose was 15 mg or less and when the patient was clear of blisters or active disease. Patients were removed from the study if they did not wish to continue for any reason, if adverse effects to azathioprine developed, if an immunosuppressive agent other than oral prednisone was added to the regimen, or if the patient's disease was deemed recalcitrant by the treating physician and the use of azathioprine was discontinued.

For statistical analysis, the Spearman rank correlation coefficient was used to summarize the strength and direction (positive or negative) of the relationship between the mean corpuscular volume (MCV) and blood levels of 6-TGN and TPMT. Regression models were fitted to evaluate the statistical significance of these relationships by means of generalized estimating equation methods that accounted for the correlation between multiple assessments on the same patient. A logarithmic transformation was applied to the 6-TGN levels before modeling. In addition, similar generalized estimating equation regression models were fitted to evaluate the relationship between aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (log transformed) and elevated levels of 6-MMP (>5700 vs ≤5700 pmol/8 × 10^8 RBCs). All calculated P values were 2-sided, and P <.05 was considered statistically significant. Statistical analyses were performed with the SAS software package (SAS Institute Inc, Cary, North Carolina).

**RESULTS**

**PATIENT CHARACTERISTICS**

A total of 27 patients were enrolled (9 men and 18 women; age range, 30-91 years). Sixteen patients (14 women and 2 men) had a diagnosis of pemphigus, 9 had bullous pemphigoid (4 women and 5 men), and 2 others (both men) had a nonimmunobullous diagnosis. Of the 16 patients with pemphigus, 1 had pemphigus foliaceus, and the others had pemphigus vulgaris. Patients were enrolled during a 2-year period and were followed up longitudinally for a median of 13.4 months.

**OPTIMAL 6-TGN LEVEL**

Data from 12 patients were used to calculate the optimal 6-TGN level (6 patients with pemphigus vulgaris, 1 with pemphigus foliaceus, and 5 with bullous pemphigoid).
The 6-TGN level was obtained during 51 follow-up visits when the patient was clear of active disease and blisters and was receiving a prednisone dosage of 15 mg/d or less and no other immunosuppressive agents.

During these 51 follow-up visits, the level of TGN ranged from 48 to 457 pmol/μL of RBCs. Four patients (all with PV or PF) had localized disease with a mean 6-TGN level of 145.3 pmol/μL of RBCs (median, 123.5 pmol/μL of RBCs; range, 48-321 pmol/μL of RBCs).

Table 1. Optimal 6-TGN Levels and Azathioprine Dosages for 12 Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y</th>
<th>Disease (Lesion Location)</th>
<th>Optimal 6-TGN, pmol/μL of RBCs</th>
<th>Total 6-TGN, pmol/μL of RBCs</th>
<th>6-MMP, pmol/μL of RBCs</th>
<th>TPMT, U/mL of RBCs</th>
<th>TPMT PCR Genotyping</th>
<th>Prednisone Dosage, mg/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/M/80</td>
<td>6</td>
<td>PV (oral)</td>
<td>380.8 (27.9-457.0)</td>
<td>350.3 (185.4-457.0)</td>
<td>14.0-171.0</td>
<td>10.2 (9.2-24.0)</td>
<td>14.8</td>
<td>HZ</td>
</tr>
<tr>
<td>5/F/79</td>
<td>5</td>
<td>PV (genital)</td>
<td>241.6 (166.0-425.0)</td>
<td>226.2 (112.0-425.0)</td>
<td>12.0-197.0</td>
<td>10.2 (9.2-24.0)</td>
<td>14.8</td>
<td>HZ</td>
</tr>
<tr>
<td>5/F/55A</td>
<td>5</td>
<td>PV (oral, genital)</td>
<td>14.0 (10.0-140.0)</td>
<td>9.6 (80.0-140.0)</td>
<td>7.0-19.0</td>
<td>14.8 (9.2-24.0)</td>
<td>14.8</td>
<td>HZ</td>
</tr>
<tr>
<td>5/F/55B</td>
<td>5</td>
<td>PV (oral, genital)</td>
<td>260.0 (184.0-321.0)</td>
<td>321.9 (62.0-321.0)</td>
<td>10.2 (9.2-24.0)</td>
<td>14.8 (9.2-24.0)</td>
<td>14.8</td>
<td>HZ</td>
</tr>
<tr>
<td>5/F/58</td>
<td>5</td>
<td>PV (oral, chest)</td>
<td>260.0 (184.0-321.0)</td>
<td>230.0 (107.0-321.0)</td>
<td>12.0-197.0</td>
<td>10.2 (9.2-24.0)</td>
<td>14.8</td>
<td>HZ</td>
</tr>
<tr>
<td>5/F/63</td>
<td>5</td>
<td>PV (oral)</td>
<td>135.2 (92.0-219.0)</td>
<td>147.9 (92.0-219.0)</td>
<td>16.2-187.0</td>
<td>14.8 (9.2-24.0)</td>
<td>14.8</td>
<td>HZ</td>
</tr>
<tr>
<td>5/F/58B</td>
<td>5</td>
<td>PV (oral, chest)</td>
<td>74.4 (49.0-137.0)</td>
<td>75.3 (49.0-137.0)</td>
<td>10.2 (9.2-24.0)</td>
<td>14.8 (9.2-24.0)</td>
<td>14.8</td>
<td>HZ</td>
</tr>
<tr>
<td>8/F/43B</td>
<td>5</td>
<td>PV (oral)</td>
<td>101.6 (48.0-129.0)</td>
<td>84.1 (48.0-129.0)</td>
<td>7.0-19.0</td>
<td>14.8 (9.2-24.0)</td>
<td>14.8</td>
<td>HZ</td>
</tr>
<tr>
<td>11/F/91</td>
<td>2</td>
<td>BP (oral)</td>
<td>230.0 (207.0-253.0)</td>
<td>230.0 (207.0-253.0)</td>
<td>10.2 (9.2-24.0)</td>
<td>14.8 (9.2-24.0)</td>
<td>14.8</td>
<td>HZ</td>
</tr>
<tr>
<td>11/F/67</td>
<td>2</td>
<td>BP (oral)</td>
<td>122.0 (108.0-138.0)</td>
<td>121.5 (96.0-159.0)</td>
<td>10.2 (9.2-24.0)</td>
<td>14.8 (9.2-24.0)</td>
<td>14.8</td>
<td>HZ</td>
</tr>
<tr>
<td>12/F/63</td>
<td>2</td>
<td>BP (oral)</td>
<td>273.6 (186.0-383.0)</td>
<td>273.0 (186.0-383.0)</td>
<td>10.2 (9.2-24.0)</td>
<td>14.8 (9.2-24.0)</td>
<td>14.8</td>
<td>HZ</td>
</tr>
<tr>
<td>13/M/58</td>
<td>2</td>
<td>BP (oral)</td>
<td>119.5 (96.0-140.0)</td>
<td>132.0 (96.0-162.0)</td>
<td>10.2 (9.2-24.0)</td>
<td>14.8 (9.2-24.0)</td>
<td>14.8</td>
<td>HZ</td>
</tr>
<tr>
<td>14/M/62</td>
<td>2</td>
<td>BP (oral)</td>
<td>101.3 (83.0-124.0)</td>
<td>111.0 (83.0-124.0)</td>
<td>10.2 (9.2-24.0)</td>
<td>14.8 (9.2-24.0)</td>
<td>14.8</td>
<td>HZ</td>
</tr>
</tbody>
</table>

Abbreviations: BP, bullous pemphigoid; PF, pemphigus foliaceus; PV, pemphigus vulgaris; RBCs, red blood cells; 6-TGN, 6-thioguanine nucleotide.

b Four patients (with PV or PF) had localized disease with a mean 6-TGN level of 145.3 pmol/μL of RBCs. For the 5 patients (patients 3, 5, 7, and 8) who had limited disease involving only the oral or genital mucosa, the face, or limited areas on the chest (Table 2), the optimal 6-TGN level for patients with limited disease ranged from 48 to 321 pmol/μL of RBCs (mean, 145.3 pmol/μL of RBCs); for patients with generalized disease, the range was 83 to 457 pmol/μL of RBCs (mean, 211.5 pmol/μL of RBCs).

The dosage of azathioprine required to maintain remission was 25 to 200 mg/d for all 12 patients (Table 1). The lowest dosage of 25 mg/d was used for a patient (patient 1) who had TPMT activity in the heterozygous range (Table 2).

Table 3. Characteristics, Laboratory Test Values, and Optimal 6-TGN Levels for 12 Patients Who Achieved Remission

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y</th>
<th>Disease (Lesion Location)</th>
<th>Optimal 6-TGN, pmol/μL of RBCs</th>
<th>Total 6-TGN, pmol/μL of RBCs</th>
<th>6-MMP, pmol/μL of RBCs</th>
<th>TPMT, U/mL of RBCs</th>
<th>TPMT PCR Genotyping</th>
<th>Prednisone Dosage, mg/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/M/25</td>
<td>20</td>
<td>BP (gen)</td>
<td>200.0 (140.0-321.0)</td>
<td>197.9 (132.0-321.0)</td>
<td>10.2 (9.2-24.0)</td>
<td>14.8 (9.2-24.0)</td>
<td>14.8</td>
<td>HZ</td>
</tr>
<tr>
<td>2/F/22</td>
<td>22</td>
<td>BP (oral, chest)</td>
<td>250.0 (162.0-321.0)</td>
<td>250.0 (162.0-321.0)</td>
<td>10.2 (9.2-24.0)</td>
<td>14.8 (9.2-24.0)</td>
<td>14.8</td>
<td>HZ</td>
</tr>
<tr>
<td>24/F/27</td>
<td>27</td>
<td>BP (oral, chest)</td>
<td>150.0 (124.0-200.0)</td>
<td>150.0 (124.0-200.0)</td>
<td>10.2 (9.2-24.0)</td>
<td>14.8 (9.2-24.0)</td>
<td>14.8</td>
<td>HZ</td>
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<tr>
<td>26/F/27</td>
<td>27</td>
<td>BP (oral, chest)</td>
<td>150.0 (124.0-200.0)</td>
<td>150.0 (124.0-200.0)</td>
<td>10.2 (9.2-24.0)</td>
<td>14.8 (9.2-24.0)</td>
<td>14.8</td>
<td>HZ</td>
</tr>
</tbody>
</table>

Abbreviations: BP, bullous pemphigoid; gen, generalized; HM, homozygote, wild type; HZ, heterozygote; 6-MMP, 6-methylmercaptopurine; ND, not done; PCR, polymerase chain reaction; PF, pemphigus foliaceus; PV, pemphigus vulgaris; RBCs, red blood cells; 6-TGN, 6-thioguanine nucleotide; TPMT, thiopurine methyltransferase.

a Induction of TPMT activity during treatment with azathioprine, calculated as the difference between the highest and lowest values of TPMT activity for each patient.

b Patients with limited disease involving only mucosa, the face, or limited areas on the chest.

c A toxic reaction developed in this patient, with the white blood cell count less than 3000/μL (to convert to number of cells × 10⁶ per liter, multiply by 0.001).
Stevens-Johnson syndrome, which was responsive to mycophenolate mofetil, but his medication was switched to azathioprine for economic reasons. Review of his 6-TGN levels showed that they generally were greater than 100 pmol. The Stevens-Johnson syndrome recurred after 8 months of treatment. At that time, the level of 6-TGN was 48 pmol and the level of 6-MMP was 59 pmol, indicating that he did not adhere to his medication regimen. Similarly, patients 20 and 22 had levels of 6-TGN less than 100 pmol despite adequate azathioprine (200 mg/d). Patient 20 had very high levels of 6-MMP and high induction of TPMT, which suggested that other metabolic pathways may have a role. Patient 22 had low levels of 6-MMP and 6-TGN, indicating that she did not adhere to her medication regimen. Patient 24 had a partial response to azathioprine, but the disease never cleared; the prednisone dosage was maintained above 20 mg/d. Patient 27 responded well to azathioprine but had 1 area under a breast that had been treated with an ablative laser and was unresponsive.

TPMT ACTIVITY

Longitudinal TPMT activity was assessed in 25 of the 27 patients (Tables 2 and 3). The TPMT phenotype was verified by PCR genotyping in 15 patients. Five patients had at least 1 value of TPMT in the heterozygous range of 5 to 13.7 U/mL of RBCs (patients 1, 2, 5, 17, and 27). Phenotypes of 3 of these patients (patients 1, 17, and 27) were confirmed to be heterozygous by PCR analysis, the phenotype of patient 5 was confirmed to be homozygous, and PCR was not performed for patient 2. The dosage of azathioprine for the 3 confirmed heterozygous patients ranged from 25 to 75 mg/d. The optimal 6-TGN level was determined in only 1 of these 3 patients (patient 1) (range, 279.0-457.0 pmol/8×10^8 RBCs; average, 380.8 pmol/8×10^8 RBCs). Patient 17 dropped out early because of abnormal liver function test results, nausea, and gastrointestinal tract intolerance. The other confirmed heterozygote (patient 27) tolerated azathioprine well but, despite very high levels of 6-TGN (up to 712 pmol/8×10^8 RBCs at 50 mg of azathioprine daily), she still had an area of active disease and required at least 20 mg of prednisone. This recalcitrant area was below the breast that had been treated with a carbon dioxide laser previously but never healed. For the 3 PCR-confirmed heterozygous patients, the mean TPMT activity was 14.0 U/mL of RBCs and the median TPMT activity was 13.9 U/mL of RBCs (Table 4).

Induction of TPMT activity during treatment with azathioprine was observed in many patients. Induction was calculated as the difference in the lowest and highest values of TPMT activity for each patient (Table 3). The value of Δ ranged from 5.1 to 14.8 U/mL of RBCs in patients with responsive disease (Table 2) and from 4.9 to 23.6 U/mL of RBCs in patients with recalcitrant disease (Table 3). For homozygous patients with unresponsive or recalcitrant disease, the Δ of 9.1 to 23.6 U/mL of RBCs was much higher than those for the patients with disease that was responsive to treatment, with a Δ of 5.1 to 12.1 U/mL of RBCs (P = .13; 2-sample t test with unequal variances).

Table 5 shows the intrapatient variation of TPMT activity from visit to visit, listed in descending order of the coefficient of variation around the mean. This variability was particularly important in patient 1, who was heterozygous, as evidenced by the TPMT range of 9.2 to 24.0 U/mL of RBCs, which spans both the homozygous and the heterozygous activity ranges (Figure 1). The

Table 3. Characteristics and Laboratory Test Values for Patients Not Included in the Calculation of Optimal 6-TGN

<table>
<thead>
<tr>
<th>Patient No./Sex/Age, y</th>
<th>Disease (Lesion Location)</th>
<th>Total 6-TGN, pmol/8×10^8 RBCs</th>
<th>6-MMP, pmol/8×10^8 RBCs</th>
<th>TPMT, U/mL of RBCs</th>
<th>TPMT PCR Genotyping</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. Mean (Range)</td>
<td>No. Range</td>
<td>No. Mean (Range)</td>
<td>Δ ±</td>
<td></td>
</tr>
<tr>
<td>4/28/F/50</td>
<td>PV (gen)</td>
<td>0 NA</td>
<td>0 NA</td>
<td>3 27.7 (24.2-30.7)</td>
<td>6.5</td>
<td>HM</td>
</tr>
<tr>
<td>10/F/89</td>
<td>BP (loc)</td>
<td>0 NA</td>
<td>0 NA</td>
<td>7 29.8 (24.6-38.9)</td>
<td>14.3</td>
<td>ND</td>
</tr>
<tr>
<td>15/F/56</td>
<td>PV (gen)</td>
<td>3 159.3 (135.0-207.0)</td>
<td>3 379.0-777.0</td>
<td>1 15.4</td>
<td>NA</td>
<td>ND</td>
</tr>
<tr>
<td>16/F/81</td>
<td>BP (gen)</td>
<td>0 NA</td>
<td>0 NA</td>
<td>1 10.7</td>
<td>NA</td>
<td>ND</td>
</tr>
<tr>
<td>17/F/81</td>
<td>BP (gen)</td>
<td>1 174.0</td>
<td>1 53.0</td>
<td>1 10.7</td>
<td>NA</td>
<td>ND</td>
</tr>
<tr>
<td>18/M/87</td>
<td>PV (gen)</td>
<td>1 127.0</td>
<td>1 670.0</td>
<td>1 17.2</td>
<td>NA</td>
<td>ND</td>
</tr>
<tr>
<td>19/M/30</td>
<td>Recurrent SJS (oral mucosa)</td>
<td>8 155.2 (48.0-301.0)</td>
<td>8 59.0-821.0</td>
<td>10 25.7 (18.8-36.4)</td>
<td>17.6</td>
<td>HM</td>
</tr>
<tr>
<td>20/F/77</td>
<td>PV (oral)</td>
<td>13 82.6 (41.0-137.0)</td>
<td>12 419.0-11,653.0</td>
<td>16 27.7 (20.6-44.2)</td>
<td>23.6</td>
<td>HM</td>
</tr>
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<td>21/F/42</td>
<td>PV (gen)</td>
<td>0 NA</td>
<td>0 NA</td>
<td>0 NA</td>
<td>NA</td>
<td>ND</td>
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<tr>
<td>22/F/50</td>
<td>PV (oral/chest)</td>
<td>5 76.2 (23.0-137.0)</td>
<td>5 374.0-1314.0</td>
<td>8 18.2 (13.7-22.8)</td>
<td>9.1</td>
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<tr>
<td>23/M/82</td>
<td>BP (gen)</td>
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<td>0 NA</td>
<td>0 NA</td>
<td>NA</td>
<td>ND</td>
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<tr>
<td>24/M/43</td>
<td>BP (gen)</td>
<td>10 245.7 (97.0-365.0)</td>
<td>10 178.0-3210.0</td>
<td>13 23.4 (17.7-28.5)</td>
<td>10.8</td>
<td>ND</td>
</tr>
<tr>
<td>25/M/60</td>
<td>Undiagnosed (gen)</td>
<td>6 50.5 (10.0-79.0)</td>
<td>6 19.0-1474.0</td>
<td>9 21.8 (17.8-27.7)</td>
<td>9.9</td>
<td>HM</td>
</tr>
<tr>
<td>26/F/48</td>
<td>PV (oral)</td>
<td>3 266.7 (118.0-374.0)</td>
<td>3 2043.0-27,076.0</td>
<td>5 23.2 (15.9-27.4)</td>
<td>11.5</td>
<td>HM</td>
</tr>
<tr>
<td>27/F/48</td>
<td>PV (gen)</td>
<td>6 418.3 (10.0-712.0)</td>
<td>6 34.0-547.0</td>
<td>6 14.2 (10.9-15.8)</td>
<td>4.9</td>
<td>HM</td>
</tr>
</tbody>
</table>

Abbreviations: AZA, azathioprine; BP, bullous pemphigoid; gen, generalized; GI, gastrointestinal; HM, homozygote, wild type; HZ, heterozygote; IVIG, intravenous immunoglobulin; LFT, liver function test; loc, localized; 6-MMP, 6-methylmercaptopurine; NA, not applicable; ND, not done; PCR, polymerase chain reaction; PV, pemphigus vulgaris; RBCs, red blood cells; SJS, Stevens-Johnson syndrome; 6-TGN, 6-thioguanine nucleotide; TPMT, thiopurine methyltransferase.

| Δ | Induction of TPMT activity during treatment with AZA, calculated as the difference between the highest and lowest values of TPMT activity for each patient. |

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use of PCR was needed to confirm the heterozygous status. For most other patients, a variability in TPMT activity of 1 to 4 U/mL of RBCs was noted.

**CORRELATION BETWEEN TPMT AND 6-TGN**

We found a mild inverse relationship (Spearman correlation, \( r = -0.24 \)) between the levels of 6-TGN and TPMT; however, this relationship was not statistically significant on the basis of fitting a regression model that took into account the correlation between multiple assessments on the same patient (\( P = .81 \)).

**6-MMP LEVELS**

Twenty-two patients had at least 1 assessment of 6-MMP (Tables 2 and 3). The levels of 6-MMP ranged from 14.0 to 27 076.0 pmol/8 \( \times \) 10^8 RBCs. In patient 20, at an azathioprine dosage of 200 mg/d, the highest level of 6-TGN was 137.0 pmol/8 \( \times \) 10^8 RBCs and the highest level of 6-MMP was 11 653.0 pmol/8 \( \times \) 10^8 RBCs. This patient required intralesional corticosteroids, and the disease was considered unresponsive. A similar pattern was observed in patient 26, although the 6-MMP level was not as high as in patient 20. In patient 22, both the 6-TGN and the 6-MMP levels were low at an azathioprine dosage of 200 mg/d.

Levels of 6-MMP greater than 5700 pmol/8 \( \times \) 10^8 RBCs have been associated with liver toxic effects. On the basis of fitting a regression model that took into account the correlation between multiple assessments on the same patient, patients with a 6-MMP level greater than 5700 pmol/8 \( \times \) 10^8 RBCs were significantly more likely to have higher ALT levels than patients with 6-MMP lev-
els of 5700 pmol/8 × 10^8 RBCs or less (P = .03). Among the 29 assessments with 6-MMP levels greater than 5700 pmol/8 × 10^8 RBCs, the median ALT was 35 U/L compared with a median of 26 U/L for the 100 assessments with 6-MMP levels of 5700 pmol/8 × 10^8 RBCs or less. However, a statistically significant relationship was not observed for AST levels between elevated and non-elevated 6-MMP levels (median AST level, 24 U/L vs 22 U/L, respectively) (P = .14).

**HEMATOLOGIC TOXICITY LEVELS OF 6-TGN**

Hematologic toxicity levels of 6-TGN metabolites were not reached during this study. Hematologic toxicity, defined as a decrease in the hemoglobin level of at least 1 g/dL (to convert to grams per liter, multiply by 10) or a decrease in the white blood cell count to less than 3000/μL (to convert to number of cells × 10^9 per liter, multiply by 0.001), occurred in only patient 7 when that patient’s white blood cell count was slightly less than 3000/μL. This patient’s optimal 6-TGN level ranged from 49 to 137 pmol/8 × 10^8 RBCs. An increase in MCV, which is known to occur with azathioprine treatment, did not correlate with increasing levels of 6-TGN (Spearman correlation, r = -0.09; P = .37). No hematologic toxic effects were observed in patients with levels of 6-TGN greater than 450 pmol/8 × 10^8 RBCs.

**COMMENT**

Azathioprine is among the most frequently used corticosteroid-sparing agents in dermatology, notably because of 30 years of experience with the drug. It is effective, and it is the most economical immunosuppressant and corticosteroid-sparing agent on the market today.

Recently, there has been interest in therapeutic blood monitoring of azathioprine metabolites for various diseases and a renewed interest in the polymorphism of TPMT. For inflammatory bowel disease, the optimal blood level of 6-TGN for therapeutic efficacy has been proposed as 235 pmol/8 × 10^8 RBCs or higher, and toxic effects have been noted at levels greater than 450 pmol/8 × 10^8 RBCs, with a proposed therapeutic range of 235 to 450 pmol/8 × 10^8 RBCs. This proposed therapeutic range has not consistently been verified. A meta-analysis of published studies on inflammatory bowel disease supported the concept that higher 6-TGN levels are associated with clinical remission. Studies of several connective tissue diseases showed no correlation between the 6-TGN levels and disease activity or remission. A kidney transplant study showed a median level of 80 pmol/8 × 10^8 RBCs (ranging from undetected to 366 pmol/8 × 10^8 RBCs).

In our study the levels of 6-TGN for inducing and maintaining remission ranged from 48 to 457 pmol/8 × 10^8 RBCs, with an optimal level at 191 pmol/8 × 10^8 RBCs for pemphigus and pemphigoid. These levels are lower than those reported for inflammatory bowel disease but are in the range reported for renal transplant. In addition, patients with localized disease, such as only mucosal involvement, can achieve remission with lower levels of 6-TGN (mean, 145 pmol/8 × 10^8 RBCs) as opposed to those with generalized disease that required higher 6-TGN concentrations (mean, 212 pmol/8 × 10^8 RBCs).

We found a mild but not significant inverse correlation between the 6-TGN level and TPMT activity. Some published studies showed a correlation and others showed no correlation between TPMT, 6-TGN, and the dose of azathioprine.

Fluctuations in TPMT of 20% around the mean, as determined by the coefficient of variation, were noted in at least 20% of our patients, which is similar to results reported by Dervieux et al. Activity of TPMT overlapping intermediate and normal activity distributions has also been reported. Increases in activity or induction of TPMT has been shown to occur with 5-aminosalicylic acid, prolonged use of thioguanine drugs, or the presence of uremia. None of our patients had uremia, nor were any of them receiving 5-aminosalicylic acid. As shown in Figure 1, TPMT variability appeared to occur as an initial induction of the activity of the enzyme most likely related to the thioguanines themselves, as previously suggested.

Patients who had large inductions of TPMT (up to 44 U/mL of RBCs, as for patient 20) had disease that was recalcitrant to treatment and therapy was considered to have failed. Table 3 shows that disease in 7 patients was recalcitrant. Six of the 7 patients were homozygous and had large inductions, with a Δ TPMT ranging from 9.1 to 23.6 U/mL of RBCs above baseline (average Δ, 14.5 U/mL of RBCs). Although similar inductions were noted in the responsive group, the Δ range for the homozygous patients was 5.1 to 12.1 U/mL of RBCs above baseline (average Δ, 9.2 U/mL of RBCs), which is much lower than the range for the unresponsive group. The mechanism of this induction may be related to the thioguanine nucleotides themselves, as has been previously reported or to other unknown mechanisms. It would seem that TPMT inductions of more than 10 U/mL of RBCs may be associated with risk of unresponsiveness.

Statistical comparison of heterozygous patients was not meaningful because there was only 1 in the respon-
Measure TPMT, U/mL of RBCs

- Deficient
  - Do not treat
- 5-11.9
  - Heterozygote
  - AZA, 25-75 mg/d
  - Confirm with PCR
- 12-14.5
  - Heterozygote or homozygote
- >14.5
  - Homozygote
  - AZA 100-250 mg/d

Measure 6-TGN and 6-MMP

- 6-TGN low
  - (<100 pmol/8×10^8 RBCs)
- 6-MMP low
- 6-TGN therapeutic
  - (100-300 pmol/8×10^8 RBCs)
- 6-MMP high

- Recalcitrant
  - Stop AZA therapy
  - Switch to another immunosuppressive agent
- Underdosed
  - Increase AZA dose
  - Repeat 6-TGN and 6-MMP
- Not adhering to treatment
  - AZA dose is already adequate

Treat with AZA

No clinical remission

- Recalcitrant disease?
  - Patient not adhering to treatment?

Clinical remission

- Continue AZA therapy
- Start prednisone taper

Figure 2. Dosing and monitoring of azathioprine (AZA) with thiopurine methyltransferase (TPMT), 6-thioguanine nucleotide (6-TGN), and 6-methylmercaptopurine (6-MMP). PCR indicates polymerase chain reaction; RBCs, red blood cells. (Note: No studies have defined high and low for 6-MMP values. With the 6-TGN therapeutic steady-state level being 300 pmol/8×10^8 RBCs, we consider 300 to 600 pmol/8×10^8 RBCs as an approximate range for 6-MMP. Values greater than this range are considered high, and values less than this range, low.)

Patients with initial TPMT activity around the cutoff of 13.7 U/mL of RBCs were also at risk of having a therapeutic failure if PCR was not performed (eg, patient 5). These patients may have been considered genotypically heterozygous and, consequently, underdosed. In this situation, PCR was helpful again. Alternatively, on the basis of the results of this study, a 6-TGN level can be measured instead of performing PCR.

In our study, occasional 6-TGN levels greater than 450 pmol/8×10^8 RBCs were reached in several patients without much evidence of toxicity. The only patient who experienced a white blood cell count less than 3000/µL had low 6-TGN levels (<100 pmol/8×10^8 RBCs). The only toxic effect noted at high levels of 6-TGN was an increase in MCV. Correlation between 6-TGN and MCV was reported by Decaux et al in 2001 but not confirmed by others. In our study, the correlation between MCV and 6-TGN was not significant (r = −0.09). We acknowledge that we did not increase the dose to the toxic range. More studies are required before the reported toxic range of greater than 450 pmol/8×10^8 RBCs can be confirmed because this upper limit has been questioned by others.

Although 6-MMP has been reported to be hepatotoxic at a level greater than 5700 pmol/8×10^8 RBCs, this has not been corroborated by others. In our study, we noted modest increases in ALT but not AST levels. In assessments with 6-MMP levels greater than 5700 pmol/8×10^8 RBCs, the median ALT level was 35 U/L compared with a median of 26 U/L for assessments with 6-MMP levels of 5700 pmol/8×10^8 RBCs or less.

Measurement of 6-MMP is valuable for detecting patients who have disease that is recalcitrant, who are not adhering to their treatment, or who are underdosed. Preferential metabolism of azathioprine to inactive metabolites rather than to active metabolites has been described. It occurs when 6-MP derived from azathioprine is preferentially metabolized to the inactive 6-MMP rather than to the active 6-TGN. The induction of TPMT as discussed earlier (particularly >10 U/mL of RBCs during treatment with azathioprine) contributes to this preferential metabolism to the inactive metabolite. A good example of this is patient 20. Even though the patient received a high dosage of azathioprine (200 mg/d), we were not able to increase or sustain the 6-TGN level, which never exceeded 137 pmol/8×10^8 RBCs, and the 6-MMP level was quite high (>11 000 pmol/8×10^8 RBCs) and was associated with an induction of TPMT up to 44 U/mL of RBCs. This is an important observation because the patient required intralesional corticosteroids and the therapy was considered to have failed. Other reasons for unresponsiveness are the lack of medication adherence or underdosing. Patient 22 received a high dosage of azathioprine (200 mg/d), but the levels of 6-TGN and 6-MMP were inappropriately low, indicating that the patient was most likely not taking her medication as prescribed. Similarly, patient 19 was also not adhering to treatment. Therapeutic levels of TGN were reached in patients 24 and 27, but both were considered to have recalcitrant disease. Both had a partial response to azathioprine. Patient 27 had ablative surgery under the breast of a pemphigus lesion that never healed, despite treatment with prednisone or azathioprine. Breast resection was recommended. Patient 24 was the only patient with truly recalcitrant disease; azathioprine was therapeutic, but previous treatment with cyclosporine and mycophenolate mofetil had failed.

In most of our patients, a therapeutic level of 6-TGN was reached about 1 to 2 months after the initiation of treatment. Attainment of a steady state has been reported to occur by 1 month after the initiation of treatment. With that information and published recommendations, we created an algorithm for monitoring azathioprine in clinical practice (Figure 2).

We recommend initiation of azathioprine therapy on the basis of the initial measurement of TPMT activity: an initial dosage of 100 mg/d for a 75-kg person (or 1.5-2
mg/kg daily) for patients with high TPMT levels in the homozygous wild-type range (14.5 U/mL of RBCs) and 25 mg/d for patients with TPMT levels in the heterozygous range. Patients deficient in TPMT should not be treated with azathioprine for dermatologic purposes unless it is the only immunosuppressive option. Patients with TPMT activity near the cutoff of 13.7 U/mL of RBCs may require confirmation with PCR to determine whether they are homozygous or heterozygous, or they may require slow increases in the azathioprine dose and monitoring of the 6-TGN level.

One to 2 months after the initiation of treatment, the 6-TGN level can be determined. This should assist in ensuring that the patient is adhering to treatment and that an optimal level is reached. It can be repeated if the dose is to be increased owing to a lack of response. When an optimal level is reached and the disease is not clinically active, the dose of corticosteroids can be tapered. Inappropriately low 6-TGN levels in a patient receiving an adequate dosage of azathioprine should alert the physician that either the patient is not adhering to treatment or the preferential pathways of metabolism are involved. When the azathioprine dose is at least 100 mg in a homozygous individual, low levels of both 6-MMP and 6-TGN indicate medication adherence issues (as in patient 26). However, a low level of 6-TGN and a high level of 6-MMP associated with an induction of TPMT activity of more than 10 U/mL of RBCs may indicate preferential pathways of metabolism to 6-MMP rather than to 6-TGN (as in patient 20). This should alert the physician that the patient will not respond adequately to any dose of azathioprine and that an alternative medication should be sought.

Levels of 6-TGN can serve as an adjunctive measure and may be helpful in the future after more data are gathered on the therapeutic range of these metabolites. Higher initial doses were recommended and shown to assist in improved outcome and decreased corticosteroid use.

The strength of our study lies in its partially blinded and prospective nature. Patients were treated by their primary dermatologist without knowledge of the 6-TGN or 6-MMP levels, and therefore they can be considered as blinded. Even though the data were available to the principal investigator, they could not be used to monitor patients. The assays were run on stored blood samples every few months; therefore, results were not always available. Decisions to modify the dose were made on the basis of patient response and laboratory-observed side effects according to current practices.

The weakness of the study was related to the same characteristic. Most physicians were reluctant to increase the dosage of azathioprine beyond the dosage they were comfortable with; therefore, a toxic level was not determined. Although we did not identify a definite therapeutic range, that can be determined from other published studies because toxicity is not disease specific.

In conclusion, our study identifies an optimal level of 6-TGN that may guide dermatologists as they monitor their patients receiving azathioprine. It may also help in allowing faster tapering of corticosteroid doses and in detecting nonresponders and patients who are not adhering to treatment.


