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Short communication

A study of the utility of azathioprine metabolite testing in myasthenia gravis

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ABSTRACT

Myasthenia gravis (MG) is an autoimmune neuromuscular disorder characterised by fatigable voluntary skeletal muscle weakness. The underlying pathogenesis is complex involving adaptive autoimmune responses. Azathioprine remains a first line broad acting immunosuppressant for MG. Due to varied clinical responses to azathioprine we aimed to investigate the relationship between azathioprine metabolites and symptom control. Mild correlations between Quantitative Myasthenia Gravis Score (QMG) vs. 6-thioguanine nucleotides ($R = -0.317$ $p = 0.186$) and QMG vs. lymphocyte count ($R = 0.402$ $p = 0.08$) were found. Azathioprine metabolite measurement should be considered in MG patients with; pancytopenia, deranged liver function or recurrent infections.

1. Introduction

Myasthenia gravis (MG) is an autoimmune neuromuscular disorder characterised by fatigable voluntary skeletal muscle weakness often caused by anti-nicotinic acetylcholine receptor autoantibodies. The underlying pathogenesis of MG is complex and involves a combination of both autoimmune adaptive humoral and cellular responses with loss of tolerance to several components of the neuromuscular junction as often implicated by the presence of specific autoantibodies (Gilhus et al., 2011; Vincent and Willcox, 1999). Considering the uncertainties surrounding the pathogenesis and clinical heterogeneity of MG, patients are frequently treated with broad acting immunosuppressive regimes including corticosteroids and steroid-sparing drugs to control symptoms.

Azathioprine is an immunosuppressant drug with randomised control trial evidence of efficacy in myasthenia gravis (Bromberg et al., 1997; Palace et al., 1998). The thiopurine drug remains the first line steroid sparing immunosuppressant for MG (Gilhus and Verschuuren, 2015). The immunosuppressive action of azathioprine is due to the generation of 6-thioguanine nucleotides (6-TGN). 6-TGN exerts immunosuppressive actions in two ways; the intracellular accumulation of 6-TGN in lymphocytes leads to incorporation of these foreign exogenous nucleotides into the cell's DNA causing cell cycle arrest and apoptosis. Secondly, the thioguanine nucleotide; 6-thioguanine-5′-triphosphate inhibits Rac1-GTPase dependent CD28 co-stimulation of T cells leading to T cell apoptosis (Tiede et al., 2003). Excessive levels of 6-TGN lead to myelosuppression via inhibition of the mitotic active of rapidly dividing haematopoietic stem cells.

6-methylmercaptopurine (6-MMP) is an inactive by-product of azathioprine metabolism by thiopurine S-methyltransferase (TPMT). Variation in the enzymatic active of TPMT within the population is common and due to frequent genetic polymorphisms (Gisbert et al., 2007). Low TPMT activity results in increased 6-TGN production and greater risk of myelosuppression. In contrast, high TPMT enzymatic activity may cause clinical non-response due to preferential metabolism to inactive 6-MMPN and a lack of immunosuppressive 6-TGN production in vivo. It is good practice to check TPMT enzymatic function or genotype in patients prior to commencing azathioprine as elevated 6-MMPN levels can cause hepatotoxicity and high 6-TGN can lead to myelosuppression.

The utility of routine measuring of the azathioprine metabolites 6-TGN and 6-MMPN in cases of inflammatory bowel disease has reported in many studies with conflicting recommendations (Amin et al., 2015; Wright et al., 2004). Azathioprine is typically prescribed at a dose of 2–2.5 mg/kg/daily for MG patients, with regular monitoring of full blood count to check for leucopenia, mean corpuscular volume and liver function tests (Heerasing et al., 2015). With the observation of varying efficacy of azathioprine in MG we aimed to investigate azathioprine metabolites in MG, which has not been reported previously. The purpose of this study was to investigate the relationship between azathioprine metabolite profiles and clinical symptom control in MG patients and provide power calculations for larger studies. Patients were

Abbreviations: 6-MMPN, 6-methyl-mercaptopurine; 6-TGN, 6-thioguanine nucleotides; MCV, mean corpuscular volume; QMG, Quantitative Myasthenia Gravis; RBC, red blood cells; TPMT, thiopurine S-methyltransferase.

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clinically assessed using the Quantitative Myasthenia Gravis Score (QMG) (Hart et al., 2007) (Barnett et al., 2012) and had blood taken for azathioprine metabolites and routine blood parameters during the same clinic visit.

2. Materials and methods

2.1. Subjects

Nineteen patients with MG were enrolled into the study. Participants had not been exposed to immunosuppressive agent other than azathioprine and prednisolone.

Inclusion criteria included:

1. A clinical diagnosis of MG with fatigable muscle weakness and
   Either
   Antibodies to anti-acetylcholine receptor and/or anti-muscle specific kinase autoantibodies.
   Or
   Single fibre electromyography demonstrating diagnostic jitter, with/without supportive progressive decline in compound muscle action potential on repetitive nerve stimulation, with/without supportive positive edrophonium test.
2. Patients were aged 18-years-old or above old at time of enrolment.
3. ≥52 weeks on azathioprine treatment and, greater than 6 weeks since a dose change, to ensure stable a stable azathioprine metabolite profile.

Exclusion criteria included:

1. Less than 52 weeks on azathioprine.
2. Plasmapheresis within 4 months of enrolment.
3. Immunoglobulin therapy within 4 months of enrolment.
4. Planned or current pregnancy and/or breast-feeding.

2.2. Quantitative Myasthenia Gravis scoring

The QMG is a peer reviewed MG clinical assessment tool. QMGs were recorded by the same individual in each case to minimise inter-observer errors. The QMG was recorded during the same visit as the blood draw for azathioprine metabolites.

2.3. Azathioprine metabolite measurement

Blood samples for 6-TGN and 6-MMPN levels were collected in ethylenediaminetetraacetic acid (EDTA) containing tubes. Acid hydrolysis was used to release 6-TGN and 6-MMPN from red blood cells and convert them to 6-thioguanine and 6-MMPN derivatives. The nucleotide derivatives were then measured using reverse phase high-performance liquid chromatography as previously described (Dervieux and Boulieu, 1998). Metabolite levels are expressed in pmol/8 × 10^8 red blood cells (RBC).

2.4. Statistical analysis

Data were analysed using SPSS 22. Data correlation was performed using 2-tailed Spearman's rank correlation for analysis.

3. Results

The MG patient cohort demographics were typical for patients attending the MG clinic with a mean age of 59.5 years, heterozygous mix of gender and generalised or ocular MG. 13/19 participants had seropositive MG. 5/19 of participants had undergone thymectomy due to thymoma. No participant had undergone thymectomy in the absence of radiological evidence of a thymoma pre-operatively (Table 1). The mean azathioprine dosage use was 180 mg/day (range 150–225 mg) and mean prednisolone dosage was 6 mg daily (range 0–30 mg) (Table 1). Blood results showed mean 6-TGN = 430.6 pmol/8 × 10^8/l RBC, mean 6-MMPN = 5535 pmol/8 × 10^8/l RBC and mean lymphocyte count = 0.85 × 10^8/l within the study cohort (Supplementary Table 1).

Table 1
MG patient cohort baseline data.

	MG patient cohort (n = 19)
Age (years)[a]	59.5 (29–83)
Female: Male (n)	13:6
Seropositive: seronegative (n)	13:6
QMG[b]	7 (0–17)
Generalised: ocular (n)	13:6
Thymoma (n)[c]	5
Thymectomy (n)[d]	5
Daily prednisolone dosage (mg)[e]	6 (0–30)
Azathioprine dosage (mg)[f]	180 (150–225)

[a] Age values are presented as mean (range).
[b] QMG are presented as mean (range).
[c] Number of the MG cohort that have/had evidence of thymoma.
[d] Number of the MG cohort that had undergone thymectomy.
[e] Daily prednisolone dosage presented as mean (range).
[f] Azathioprine dosage presented as mean (range).

The correlation observed between QMG and 6-TGN had a correlation coefficient (R) = −0.317, p = 0.186, QMG vs. 6-MMPN R = −0.27, p = 0.912, QMG vs. MCV R = −0.51, p = 0.835 and QMG vs. lymphocyte count R = 0.402, p = 0.08 (Fig. 1). The correlation between lymphocytes and 6-TGN was R = −0.25, p = 0.303 and lymphocytes vs. 6-MMPN R = −0.399, p = 0.091. This study was underpowered to demonstrate statistical significances (defined as $p < 0.05$). The mild correlation (R = −0.317) between QMG and 6-TGN reported predicts a required sample size of 76 patients to achieve a study with 80% power. Similarly, the correlation (R = 0.402) between lymphocyte count and QMG indicates that a sample size of 47 participants would be required to achieve a study with 80% power.

Participant 01 (Supplementary Table 2, number 01) with elevated levels of 6-TGN at 730 pmol/8 × 10^8/l RBC and 6-MMPN of 32,873 pmol/8 × 10^8/l RBC had a history of recurrent cellulitis with bacterial infection including pseudomonas. During azathioprine treatment he had developed pancytopenia with haemoglobin of 89 g/l, MCV 110 fl, white cell count 1.2 × 10^9/l, neutrophils 0.8 × 10^9/l, lymphocytes 0.3 × 10^9/l and platelets of 167 × 10^9/l. A reduction in his azathioprine dosage from 225 mg/day (2.1 mg/kg/day) to 125 mg/day (1.2 mg/kg/day) resulted in an improvement in his blood counts within 1 month to a haemoglobin of 103 g/l, white cell count 5.9 × 10^9/l, neutrophils 5 × 10^9/l, lymphocytes 0.5 × 10^9/l and platelets 200 × 10^9/l. This coincided with resolution in infections with no worsening of MG symptoms.

4. Discussion

In this study the most significant correlation observed was the between clinical symptom control (as assessed by QMG) and lymphocyte count. This raises some interesting questions as to the underlying pathological mechanism of MG as well as whether future treatment strategies should be directed towards the adaptive cellular immune response (Dalakas, 2015). A lower total lymphocyte count and increased MCV have mild correlations with improved symptom control in MG patients treated with azathioprine. This observation of lymphocyte depletion in MG patients is supported by the evidence of a pathogenic role of T helper-17 cells and dysregulated thymic T cell selection in MG (Wang et al., 2015) (Gradolatto et al., 2014). This symptom improvement may not be directly attributable to a reduction in lymphocyte numbers, as T cell specific immunosuppressive treatment impairing T cell effector functions and not absolute T cell numbers have also shown benefit in MG control (Cruz et al., 2015) (Dalakas, 2015). The correlation between reduced lymphocyte count and improved QMG may support