Is the Oral Methionine Loading Test Insensitive to the Remethylation Pathway of Homocysteine?

To the Editor:

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in the remethylation pathway of homocysteine. The study by Girelli et al.\(^1\) showed that the C677T mutation of the gene encoding for MTHFR is common in Italy, is not associated with coronary atherosclerotic disease, and influences the plasma levels of total homocysteine (tHcy) only in subjects who have low serum concentrations of folic acid. These findings are important confirmations of previous reports.\(^2,6\) In addition to the above issues, Girelli and his coworkers addressed the problem of the influence of the MTHFR genotype on the increases of plasma tHcy levels after an oral methionine loading. The methionine loading test, which was originally developed to detect heterozygosity for the deficiency of cystathionine-β-synthase, a key enzyme in the trans-sulfuration pathway of homocysteine,\(^7\) is useful not only to detect subjects with defects of the trans-sulfuration pathway, but also to identify subjects at risk for thrombosis.\(^8,9\) It has been suggested that abnormal post-methionine loading (PML) increases in the plasma levels of tHcy reflect abnormalities of the trans-sulfuration pathway of homocysteine metabolism and are insensitive to the remethylation pathway, in contrast with fasting plasma levels of tHcy, which are sensitive to the remethylation pathway.\(^10,11\) If this hypothesis were true, the T/T mutation at nucleotide 677 of MTHFR, which is associated with an enzyme activity of about 50% of normal, should not influence the results of the methionine loading test. Girelli et al commented that their results agree with this hypothesis, because they showed that heterozygosity for the C677T mutation of MTHFR is not associated with abnormally high PML tHcy increments.\(^1\) However, they found higher PML tHcy increments in individuals carrying the mutant T/T genotype (25.5 µmol/L) than in those with normal (C/C) or heterozygous (C/T) genotypes (23.6 µmol/L for both), although the difference was not statistically significant (Table 1). In addition, they found that individuals with low serum levels of folate, which plays an important role in the remethylation pathway, tended to have higher PML tHcy increments than those with high folate levels, independently of their genotype (24.9 vs. 22.9 for C/C, 25.1 vs. 23.5 for C/T, 28.8 vs. 21.4 for T/T). In our opinion, these data do not allow a definite conclusion regarding the influence of defects of the remethylation pathway on the methionine loading test. At least four additional reports with an experimental design similar to that of Girelli et al have been published thus far.\(^2,3,5,6\) All of them clearly showed that the fasting tHcy levels in plasma are significantly higher in the T/T genotype of MTHFR than in the other genotypes; however, the results of the methionine loading test were less consistent. Table 1 shows that all the studies, perhaps with the exception of that by Jacques et al,\(^4\) showed that the PML increases of tHcy in plasma were higher in the T/T genotype of MTHFR than in the normal C/C genotype, although the statistical significance was reached in only two.\(^2,5\) Since the publication of our study in 1997,\(^2\) we screened more individuals for MTHFR genotype and the methionine loading test. Our most recent analysis of the data obtained in 399 healthy controls and 250 patients with previous episodes of arterial or venous thrombosis confirmed our previous findings that the PML increases of tHcy levels in plasma are significantly higher in individuals with the T/T genotype (18.2 ± 7.5, arithmetic mean ± SD) than in those with the C/C or the C/T genotypes of MTHFR (14.1 ± 5.6, P < .001). The differences remained highly statistically significant (P < .001) also after adjustment for the serum levels of folate and cobalamin, and the plasma levels of vitamin B6, which is the essential cofactor in the trans-sulfuration pathway of homocysteine.\(^3\) Among the 649 subjects studied, 142 had the T/T genotype of MTHFR (80 controls and 62 cases); 14 of them (9.9%) had high fasting tHcy levels, 15 (10.6%) had both high fasting levels and high PML increases of tHcy, and 6 (4.2%) had high PML increases of tHcy. Therefore, the methionine loading test allowed the identification of 6 additional subjects with the MTHFR variant who had normal fasting levels of tHcy.

In conclusion, the available experimental evidence indicates that the fasting levels of tHcy in plasma are very sensitive to an abnormality of the remethylation pathway of homocysteine, such as that associated with the T/T genotype of MTHFR; however, they do not allow the conclusion that the PML increases of tHcy levels in plasma only reflect abnormalities of the trans-sulfuration pathway of homocysteine, because they also tend to be abnormal in individuals with the T/T genotype of MTHFR and in those with low serum levels of folic acid.

Marco Cattaneo
Rossana Lombardi
Anna Lecchi
Maddalena L. Zighetti
A. Bianchi Bonomi Hemophilia and Thrombosis Center
Department of Internal Medicine
IRCCS Ospedale Maggiore
University of Milano
Milano, Italy

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Table 1. Increases of Plasma tHcy After an Oral Methionine Loading as a Function of MTHFR Genotypes

<table>
<thead>
<tr>
<th>Study</th>
<th>No. of Subjects</th>
<th>MTHFR Genotype at nt 677</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>C/C</td>
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<tr>
<td></td>
<td></td>
<td>C/T</td>
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<tr>
<td></td>
<td></td>
<td>T/T</td>
</tr>
<tr>
<td>Jacques et al,</td>
<td>365</td>
<td>17.9</td>
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<tr>
<td>1996†</td>
<td></td>
<td>18.5</td>
</tr>
<tr>
<td>Cattaneo et al,</td>
<td>231</td>
<td>14.4</td>
</tr>
<tr>
<td>1997†</td>
<td></td>
<td>14.8</td>
</tr>
<tr>
<td>Legnani et al,</td>
<td>63</td>
<td>17.0</td>
</tr>
<tr>
<td>1997†</td>
<td></td>
<td>19.0</td>
</tr>
<tr>
<td>Verhoef et al,</td>
<td>318</td>
<td>23.6</td>
</tr>
<tr>
<td>1997†</td>
<td></td>
<td>26.4</td>
</tr>
<tr>
<td>Girelli et al,</td>
<td>415</td>
<td>23.6</td>
</tr>
<tr>
<td>1998‡</td>
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<td>23.6</td>
</tr>
</tbody>
</table>

Values of PML tHcy increase are arithmetic means (†) or geometric means (‡). The dose of oral methionine was 3.8 g/m² body surface area in the study by Cattaneo et al, and 100 mg/kg body weight in the other studies.

Abbreviation: NS, not significant.

* Genotype T/T v genotype C/C.
Response

We appreciated the comments of Dr Cattaneo et al, which raise the interesting problem of the factors influencing the PML hyperhomocysteinemia, although it was not the main object of our study.1 We also cited the only two studies published at that moment on the relationship between the C677T mutation and PML tHcy levels,2,3 pointing out their conflicting results. The thorough update by Dr Cattaneo et al adds uncertainty to this matter. The discrepancies may be partly caused by differences in the duration of PML tests (4 hours2,4,5 or 6 hours,1,3 reflecting a different stage in plasma tHcy clearance) and, more remarkably, in the statistical analysis used. For example, we1 and others2,3 compared the three MTHFR genotypes by one-way ANOVA followed by Tukey’s post-hoc comparison of the means, which is a more conservative approach. On the other hand, Cattaneo et al4 used the t-test between the T/T group and the C/C plus C/T combined group. We also revalued an increased number of subjects (n = 517), confirming the trend toward higher PML increases in the T/T group than in other genotypes, again without reaching the statistical significance (23.3, 23.6, and 25.9 µmol/L in C/C, C/T, and T/T, respectively; ANOVA: F = 1.9, P = .15; Tukey’s test: T/T v C/T, P = .19; T/T v C/C, P = .15). In our report, we concluded that our results were consistent with the generally accepted view that an abnormal PML increase in tHcy levels would primarily reflect abnormalities in the transsulfuration pathway. However, this does not exclude in principle an association between an impaired remethylation and the PML tHcy levels, whereas it is clearly not as strong as that observed with fasting Hcy. This could be due to at least two reasons. First, marginal deficiencies of folate and vitamin B6 are often correlated,6 so that if you have an impaired remethylation because of inadequate folate status, probably you will also see an impaired transsulfuration. Second, according to Sellhub and Miller’s hypothesis,7 remethylation and transsulfuration are coordinately regulated so that a defect in one pathway will lead to the impairment of the other. A key role is played by S-adenosylmethionine (SAM), the most important intermediate in the synthesis of homocysteine from methionine, which is both an allosteric inhibitor of MTHFR and an activator of cystathionine-β-synthase (CBS). In subjects with fasting hyperhomocysteinemia because of a primary impairment of the remethylation pathway (ie, defective MTHFR, folate deficiency, or both) the decreased de novo synthesis of methionine implies a decreased cellular SAM concentration, ultimately leading to a suboptimal activation of CBS. So, it is conceivable that these subjects also tend to have slightly increased PML tHcy value because of a basal defect in the transsulfuration, which is partly counterbalanced by the PML-induced increase of SAM. However, from a practical standpoint this phenomenon may not be so relevant, as the determination of fasting tHcy is clearly sufficient to detect subjects with abnormalities in the remethylation. Thinking in terms of vascular risk, the PML test is important mainly for detecting subjects with isolated PML hyperhomocysteinemia, ie, a subject who could be at risk but would not be diagnosed with fasting tHcy determination alone. It was previously reported that this subgroup may represent up to 40%6 of the whole hyperhomocysteinemic population, and our recent analysis9 is in keeping with this view. In our opinion, it is especially in this subgroup that we need to learn much more about the determinants of PML tHcy by means of further comprehensive studies including all potential genetic and environmental factors (CBS polymorphisms, vitamin B6 status, and others).

Domenico Girelli
Oliviero Olivieri
Carla Russo
Roberto Corrocher
Department of Clinical and Experimental Medicine
University of Verona
Verona, Italy

REFERENCES

intracellular cytokine profiles of cord and adult blood lymphocytes.

To the Editor:

In a recent issue of Blood, Chalmers et al1 communicated their findings of intracellular cytokine profiles of cord and adult blood. Analyzing cytokine synthesis at a single-cell level, they showed that cord blood (CB) lymphocytes produced less interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) than adult peripheral blood (AB) lymphocytes. Further subset analysis indicated that in CB lymphocytes, the majority of cytokine-producing cells were CD4+ CD45RA+ in contrast to AB lymphocytes, which were both CD4+ CD45RO+ and CD8+ CD45RO+. Thus, they concluded that the reduced incidence of graft-versus-host disease (GVHD) in CB stem cell transplantation (CBSCT) could be partly due to the observed altered cytokine profile in CB lymphocytes.

Interestingly, we also recently conducted a similar study assessing intracellular cytokine profiles of purified CB CD4+ and CD8+ T-cell subsets in comparison with those of healthy older children and adults. We have found and reported IFN-γ production capability to be altered and significantly lower (<5% of total T cells) in CB compared with that of older children and adults.2 However, in contrast, our findings revealed normal and comparatively high IL-2 production capability, at a single-cell level, relative to older children and adults with even significantly higher mean cell percentage values of the cytokine-producing cell populations in CB. In a concurrent assessment of CB mononuclear cell population able to produce IFN-γ, we found that a large proportion of natural killer (NK) cells had the ability to produce IFN-γ with virtually no CD4+ cells (<1%) being able to do so (Fig 1). Thus, the few IFN-γ-producing CB mononuclear cells were almost exclusively not T but largely NK cells. We observed that CB T lymphocytes, as indicated by their almost exclusive expression of CD45RA antigen and sole production of IL-2, were comprised almost exclusively of T helper (Th) and T cytotoxic (Tc) precursor cells, explaining in part the observed altered low IFN-γ production. However, when CB mononuclear cells were cultured with anti-CD3 monoclonal antibody and exogenous IL-2, the IFN-γ-producing cells markedly increased, reaching levels comparable to those of AB (Fig 2). This suggests that CB T cells may not, as such, be hyporesponsive in an in vivo alloantigen setting such as during CBSCT. Indeed, recent reports1,4 together with our in vitro studies (manuscript in preparation) suggest that the neonatal T cell, though naive, is immunologically and comparatively competent. Thus, factors other than altered cytokine profiles, not yet sought and investigated, may fully explain the reported infrequent GVHD associated with CBSCT.

Finally and understandably, the variation in intracellular IL-2 and IFN-γ values between the report of Chalmers et al and our report, among other factors, could be due to intracellular staining protocol modifications. Indeed, Jason and Larned5 have recently illustrated how slight protocol modification in intracellular cytokine flow cytometry can lead to diverse variations of fluorescence stain results. Chalmers et al used 5 ng/mL phorbol 12-myristate 13-acetate (PMA), whereas we used 50 mM (30.8 ng/mL) to induce cytokine production. It is documented that a high concentration of PMA may be necessary to induce a more profound T-cell response, such as the expression of IL-2 receptor.6 However, despite the use of a high concentration of PMA in our study, CB IFN-γ-producing cells were consistently low in both CD4+ and CD8+ T-cell subsets.

James Chipeta
Yoshihito Komada
Xiao-Li Zhang
Minoru Sakurai
Department of Pediatrics
Eiichi Azuma
Department of Clinical Immunology
Mie University School of Medicine
Mie, Japan

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