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Genetic Effects on Variation in Red-Blood-Cell Folate in Adults: Implications for the Familial Aggregation of Neural Tube Defects

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Summary

Recent studies have implicated folic acid as an important determinant of normal human growth, development, and function. Insufficient folate levels appear to be a risk factor for neural tube defects (NTD), as well as for several chronic diseases of adulthood. However, relatively little is known about the factors that influence folate status in the general population. To estimate the relative contribution of genetic and nongenetic factors to variation in folate, we have evaluated red blood cell (RBC) folate levels in 440 pairs of MZ twins and in 331 pairs of DZ twins. The data were best described by a model in which 46% of the variance in RBC folate was attributable to additive genetic effects, 16% of the variance was due to measured phenotypic covariates, and 38% of the variance was due to random environmental effects. Moreover, the correlations for RBC folate in MZ co-twins (r = .46) and in repeat measures from the same individual (r = .51) were very similar, indicating that virtually all repeatable variation in RBC folate is attributable to genetic factors. On the basis of these results, it would seem reasonable to initiate a search for the specific genes that influence RBC folate levels in the general population. Such genes ultimately may be used to identify individuals at increased risk for NTD and other folate-related diseases.

Introduction

It is becoming increasingly evident that nutritional factors play an important role in health maintenance and disease prevention. The essential role of nutrients in maintaining normal human growth, development, and function is dramatically illustrated by recent studies that demonstrate that maternal periconceptional supplementation with folic acid is protective against fetal neural tube defects (NTD) (MRC 1991; Czeizel and Dudas 1992). The risk of orofacial clefts, congenital heart defects, urinary-tract anomalies, and limb abnormalities also may be reduced by such supplementation (Shaw et al. 1994, 1995; Li et al. 1995; Czeizel 1996). Moreover, there is an accumulating body of data that suggest that insufficient folate levels may be a predisposing factor for several chronic diseases of adulthood, including arteriosclerotic vascular disease (Boushey et al. 1995) and cancers of the cervix, colon, bronchus, and esophagus (Butterworth 1991).

Folic acid is a widely available, water-soluble vitamin, which acts as a cofactor for enzymes involved in RNA and DNA biosynthesis and as a methyl donor in the methylation cycle (Scott et al. 1994). Normal function is dependent on adequate dietary intake and/or supplementation, as well as on proper transport and metabolism of folic acid. The identification of several inherited disorders of folate transport and metabolism (Rosenblatt 1989) suggests that folate status is likely to be under considerable genetic control. However, relatively little is known about the genetic contribution to folate status in normal individuals.

Using a classic twin-study design, we recently have demonstrated that ~40% of the variance in red blood cell (RBC) folate levels in adult females is attributable to familial factors, with the remaining variance due to the effects of an individual's unique (i.e., nonshared) environment (Mitchell et al. 1994). However, this pilot study lacked sufficient power to differentiate between models in which the familial contribution to RBC folate was attributed to additive genetic effects only, nongenetic familial factors only, or a combination of additive genetic and nongenetic familial factors. The present, larger study was undertaken in an attempt to (i) resolve the relative contribution of genetic and nongenetic familial factors to variance in RBC folate levels and (ii) evaluate the impact that familial correlations for RBC folate have on the familial aggregation of NTD.

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Subjects and Methods

Study Subjects

The study sample was drawn from the Australian National Health and Medical Research Council Twin Registry (ATR). The ATR is a volunteer-based registry whose members are recruited through media advertisements, schools, and other community-based organizations throughout Australia. In 1980, it was estimated that the ATR included 10% of all Australian twins. The twins in this registry have been shown to be representative of the Australian population, for a number of variables, including drinking behavior, personality factors, anxiety, and depression (Treloar et al. 1992).

The present study is based on a subset of the ATR, ascertained as part of a study on the persistence and change in patterns of alcohol use. Six thousand eight hundred eighteen twins born prior to 1964 were invited to participate in this study. In the first phase of the study, each subject participated in an extensive telephone interview that was designed to assess alcohol problems and other psychiatric symptoms. Subjects also were asked to assess the frequency with which they saw or had other contact (e.g., via telephone or letter) with their co-twin. Information on dietary practices relating to folate status were not collected as part of this study.

Interviews were conducted during 1992 and 1993 and were completed by 5,995 (87.9%) of the eligible twins. Interviews were not completed for the following reasons: lost to follow-up (n = 148 [2.2%]), located after completion of the interview phase of the study (n = 13 [0.2%]), deceased (n = 78 [1.1%]), withdrawn from the registry (n = 30 [0.4%]), or refused to participate (n = 554 [8.1%]).

In the second phase of the study, a subset of the subjects who completed the telephone interview were asked to provide blood samples for genetic and biochemical analyses. The only restriction placed on subjects in this phase of the study was that they had to reside in the vicinity of one of five major Australian cities (Adelaide, Brisbane, Canberra, Melbourne, or Sydney). Hence, this subset of subjects should be random with respect to RBC folate. Blood samples were obtained at clinics held in each of these cities, during visits to the subject's home, or by mail. Each subject was asked to provide 40 ml of venous blood. In addition, information was collected on the type and quantity of alcohol consumed in the 7 d preceding blood collection. Blood samples were obtained for 3,348 study subjects, over a 20-mo period from August 1993 to March 1995.

Blood samples collected at the clinics and during home visits (n = 2,416) were refrigerated and processed within 2 h. The processed samples, including 5 ml of packed, unwashed, RBCs obtained from blood collected into EDTA tubes, were kept at −20°C and subsequently were transported, on dry ice, to a central biochemistry laboratory in Brisbane. A subset of the RBCs (n = 895) obtained during the 1st year of data collection initially were stored at −20°C and were transferred to −70°C storage at the end of 1994. All other samples were stored at −70°C. Samples obtained by mail (n = 932) were excluded from the present study, because of concerns regarding folate deterioration during sample transport.

Zygosity Assignment

The zygosity of twins in this sample was assigned on the basis of a standard zygosity questionnaire and, in ambiguous cases, by examination of photographs. In the ATR, this classification scheme has been shown to provide ≥98% agreement with zygosity diagnoses made on the basis of extensive blood typing (N.G.M. and D.L.D., unpublished data).

Folate Assays

Folate levels were assessed in RBCs rather than in plasma, because the former is a more stable indicator of folate status (Leck 1977). In addition, the risk of NTD has been more consistently associated with maternal RBC folate than with maternal plasma folate (Wald 1993).

RBC folate concentrations were measured by use of an automated chemiluminescence system (ACS-180; Ciba-Corning). This assay measures N^5-methylenetetrahydrofolate, the primary form of circulating folate (Scott et al. 1994). The assays were performed according to the recommended procedures for this system, with 25 μl of thawed RBCs diluted in 500 μl of ascorbic acid.

The study samples were analyzed in three randomly selected groups, and the samples in each group were assayed in batches during 2-wk intervals in August 1995, November 1995, and April 1996. Intra-assay variability, estimated from duplicate samples assayed in the same batch (n = 101), was 19%. The relatively low folate values (mean 125.6 ng/ml, SD 34.1) reported in this study are likely to reflect sample dilution due to residual serum within the packed RBCs.

Statistical Methods

Preliminary statistical analyses were performed by use of SAS 6.10 (SAS Institute 1991). Nonparametric procedures were used in these analyses, since the null hypothesis of normality was rejected in this sample (Kolomogorov test; P = .02).

Evaluation of the relative contribution of genetic and nongenetic factors to variation in RBC folate was accomplished by use of the computer program FISHER (Lange et al. 1988). This program was designed for the analysis of classical biometric traits and performs likeli-

hood calculations under the assumption that the measured phenotype conforms to a multivariate normal distribution. With this program it is possible to specify the expected phenotypic value of an individual, in terms of the fixed effects of variables such as age and sex. Both variance about these means and covariance between relatives then can be specified in terms of either the twin correlations or environmental and genetic components of variance.

Models of variation were fitted directly to the observed RBC folate data. Hypotheses regarding the significance of model parameters were evaluated by use of the likelihood-ratio test—that is, twice the difference between the log likelihood of models with and without the parameter(s) of interest. This test is distributed approximately as a χ^2, with the df determined by the difference in the number of parameters estimated by the two models.

The method of Hopper and Carlin (1992) was used to evaluate the potential contribution that familial correlations for RBC folate make to the familial aggregation of NTD. This method quantifies the relations between (i) the intrapair correlation for a quantitative risk factor in a randomly selected pair of relatives, (ii) the association between the quantitative risk factor and the probability of being affected, and (iii) the resulting disease association between relatives, represented as an odds ratio. Under this model, the risk of disease is assumed to be a logistic function of the risk factor and is summarized by the interquartile disease-risk ratio (i.e., the risk to individuals in the upper quartile of the distribution, compared with the risk to individuals falling in the lower quartile, for the quantitative risk factor).

Results

The study sample comprised 2,207 Caucasian individuals—1,418 females and 789 males. The study subjects had an age range of 30–86 years and represented 771 complete twin pairs (i.e., twin pairs for whom RBC folate data were available on both members) and 665 individuals whose co-twins were not available for study. The average interval between collection of venous blood samples and evaluation of RBC folate levels was 463.9 d (range 250–826 d).

The mean RBC folate values of MZ male (MZM), MZ female (MZF), DZ male (DZM), DZ female (DZF), and opposite-sex DZ twins (DZO) were not significantly different ($P = .42$). Moreover, despite significant differences in the frequency of contact between MZ and DZ co-twins ($P < .0001$), there was no evidence that the absolute intrapair difference in RBC folate levels was significantly influenced by frequency of contact (for MZ, $P = .88$; for DZ, $P = .64$). There was, however, a significant correlation ($r = .13$, $P = .008$) between the absolute MZ intrapair difference and the intrapair sum for folate, indicating that the joint distribution of twin pairs for RBC folate was not bivariate normal. This interaction is likely to be an artifact of scale, since it was removed by square-root transformation of the data ($r = -.02$, $P = .71$).

Analyses of the relative contribution of genetic and nongenetic factors to variation in RBC folate were restricted to the 771 pairs for whom RBC folate data were available on both members. These analyses were performed on the square root–transformed RBC folate data. The full-analysis model included 20 parameters: five twin correlations (r_{MZF}, r_{MZM}, r_{DZF}, r_{DZM}, and r_{DZO}); a grand mean (μ); two sex-specific variances (V_F and V_M); regression terms for the mean effects of sex, age, storage temperature, storage length, and alcohol intake; five dummy variables to account for mean differences in RBC folates from the six blood-collection sites (Adelaide, Brisbane, Canberra, Melbourne, Sydney, or home visit); and two dummy variables to account for mean differences between the three groups of folate assays (i.e., August 1995, November 1995, and April 1996). Alcohol intake was considered as a potential covariate of RBC folate, since, at least when it is consumed to excess, it interferes with folate metabolism (Davis 1986).

Results of model fitting to the RBC folate data are summarized in table 1. The hypothesis of no sex-specific differences in the variance of RBC folate (model I) could not be rejected in these data ($P = .44$). Hence, the following relationships were set in all subsequent models: $V_F = V_M$, $r_{MZF} = r_{MZM}$, and $r_{DZF} = r_{DZM} = r_{DZO}$. The hypothesis that variance in RBC folate is attributable to common familial and random environmental factors was evaluated by equating the MZ and DZ correlations (model II). This hypothesis was clearly rejected ($P = .002$). In contrast, the hypothesis that variance in RBC folate is attributable to additive genetic and random environmental factors, which was evaluated by constraining the DZ twin correlation to be one-half of the MZ twin correlation (model III), could not be rejected ($P = .64$). Hence, these data indicate that variance in RBC folate is not significantly influenced by environmental factors that are shared by co-twins.

Hypotheses regarding the significance of the phenotypic covariates (e.g., age, sex, and storage time) were tested, individually, by setting the relevant regression parameters to 0. These models were evaluated, relative to model III, by use of the likelihood-ratio test. Age ($P = .0008$), storage temperature ($P < .0001$), and blood-collection site ($P = .0024$) were significantly related to mean RBC folate, whereas sex ($P = .96$), group ($P = .39$), alcohol intake ($P = .18$), and storage time ($P = .49$) were not. The fit of the model in which the

Table 1

Parameter Estimates ± SE, under the Full-Analysis Model and under Selected Reduced Models

	Full Analysis	Model I[a]	Model II[b]	Model III[c]
μ[d]	9.89 ± .32	9.87 ± .32	9.85 ± .32	9.86 ± .32
V_F	2.01 ± .09	1.96 ± .08	1.96 ± .08	1.96 ± .08
V_m	1.86 ± .13	1.96[e]	1.96[e]	1.96[e]
r_{MZF}	.42 ± .04	.46 ± .04	.35 ± .03	.46 ± .03
r_{MZM}	.57 ± .05	.46[e]	.35[e]	.46[e]
r_{DZF}	.17 ± .08	.20 ± .05	.35[e]	.23[f]
r_{DZM}	.29 ± .12	.20[e]	.35[e]	.23[e]
r_{DZO}	.19 ± .09	.20[e]	.35[e]	.23[e]
Sex	−.003 ± .087	−.007 ± .087	−.006 ± .084	−.006 ± .086
Age	.018 ± .004	.018 ± .004	.018 ± .004	.018 ± .004
−2lnL	1,225.410	1,229.157	1,238.870	1,229.371
χ^2 (df)		3.747 (4)[g]	9.713 (1)[h]	.214 (1)[h]

NOTE.—Each model included terms for the mean effects of the blood-collection site, assay group, storage time and temperature, and alcohol use.

[a] H_0: no sex effects; $r_{MZF} = r_{MZM}$, $r_{DZF} = r_{DZM} = r_{DZO}$, and $V_F = V_M$.

[b] H_0: no additive genetic effects; $r_{MZF} = r_{MZM} = r_{DZF} = r_{DZM} = r_{DZO}$, and $V_F = V_M$.

[c] H_0: no shared environmental effects; $r_{MZF} = r_{MZM} = .5(r_{DZF}) = .5(r_{DZM}) = .5(r_{DZO})$, and $V_F = V_M$.

[d] Mean value of the RBC folate (ng/ml) values after square-root transformation.

[e] Parameter constrained to equal the parameter above.

[f] Parameter constrained to equal ½ the parameter above.

[g] Model I vs. full-analysis model.

[h] Evaluated relative to model I.

regression coefficients for all of the nonsignificant covariates were set to 0 was not significantly different from the fit of model III ($P = .45$).

Therefore, the best model for these data includes additive genetic and random environmental effects plus the effects of age, blood-collection site, and storage temperature. Under this model, ~46% of the variance in RBC folate is attributable to additive genetic effects, 2% is due to age, 14% is due to sample handling (i.e., storage temperature and blood-collection site), and 38% is due to unique environmental factors. Approximately one-half of the variance due to unique environmental factors appears to be attributable to assay variation (i.e., intra-assay variability = .19).

Test-retest correlations for 255 individuals who had their RBC folate levels assessed as part of both our pilot study and the present study were significantly greater than 0 (females—$r = .51$, 95% confidence interval [CI] .40–.60; males—$r = .51$, 95% CI .24–.71). On average, the interval between collection of the two samples was 1.9 years (range 0.3–3.5 years). The observed test-retest correlations are in good agreement with the reported correlation between maternal RBC folate levels during the first trimester of pregnancy and those 1 year later ($r = .53$) (Leck 1977). In addition, they are remarkably similar to the MZ twin correlation, indicating that virtually all of the repeatable variation in RBC folate is attributable to additive genetic factors.

Daly et al. (1995) have demonstrated that the relationship between maternal RBC folate and the risk of NTD is well described by a logistic function. Hence, the method of Hopper and Carlin (1992) was used to assess the impact that familial correlations for RBC folate may have on the familial aggregation of NTD. The NTD risk ratio between the upper and lower quartiles of RBC folate was estimated to be 3.7, on the basis of the data reported by Daly et al. (1995, table 2). In addition, the test-retest correlation estimated from the present study was used as a measure of the between-sibling correlation in prenatal exposure to maternal RBC folate levels, and the DZ twin correlation was used as a measure of the correlation between the offspring of sister-sister pairs (i.e., maternal first-cousins related through females).

Table 1 in the work of Hopper and Carlin (1992) indicates that, for a disease risk ratio of 3.7, a between-relatives correlation of .51 (i.e., the test-retest correlation) can account for a disease odds ratio of ~1.10 and that a correlation of .23 (i.e., the DZ-twin correlation for RBC folate under model III) can account for a disease odds ratio of ~1.04. (Similar results were obtained when analyses were restricted to correlations for females of reproductive age [i.e., <46 years].) However, if it is assumed that the prevalence of NTD is ~1/1,000 (after adjustment for prenatal diagnosis and elective termination) (Cragan et al. 1995), the odds of NTD in the siblings and maternal first cousins of affected individuals are increased by ~30-fold and ~10-fold, respectively (Zackai et al. 1978; Cowchock et al. 1980). Hence, familial correlations for RBC folate appear to be largely insufficient for the purposes of explaining the observed familial aggregation of NTD.

Discussion

The present study confirms our earlier report, which had indicated that there was a significant familial contribution to RBC folate in adult females (Mitchell et al. 1994), and it extends this finding to adult males. Moreover, this study indicates that the familial contribution to RBC folate is genetic in nature, with additive genetic factors accounting for ~46% of the variance in this trait among adults. Shared environmental experiences do not appear to be significant determinants of variation in adult RBC folate levels.

The true contribution of additive genetic factors to variance in RBC folate levels may be deflated by the large amount of variance due to assay error and storage

effects. Assay variability accounts for ~19% of the variance in RBC folate levels and is incorporated into the estimate of the variance attributable to unique environmental factors. Hence, only ~19% of the variance in RBC folate is likely to result from an individual's life style. Biologically relevant sources, therefore, account for only 67% of the variance in RBC folate (i.e., additive variance accounts for 46%, age effects accounts for 2%, and unique environment accounts for 19%). If only the biologically interesting sources are considered, 69% of the variance in RBC folate is attributable to additive genetic factors, 3% to age, and 28% to an individual's unique life style.

The contribution of additive genetic factors to variation in adult RBC folate levels may be overestimated if MZ twins share a more similar environment or "life style" than do DZ twins. However, there was no evidence that the frequency of contact between co-twins, which can be taken as a crude indicator of shared environment, was associated with the degree of similarity for RBC folate.

The available data suggest that familial correlations for RBC folate can account for only a small proportion of the increased NTD risk observed among relatives of affected individuals. Because of the lack of data regarding the functional relationship between RBC folate and the risk for other diseases (e.g., arterosclerotic vascular disease), it was not possible to assess the impact that familial correlations for RBC folate have on the familial aggregation of these conditions. However, it has been demonstrated that, in general, polygenic inheritance of continuously distributed risk factors can account for only modest familial aggregation of disease (Aalen 1991; Hopper and Carlin 1992).

On the basis of the results from the present study, it would seem reasonable to initiate a search for the specific genes that influence RBC folate levels in the general population. Such genes ultimately may be used to identify individuals who are at increased risk for specific folate-related diseases. In addition, rare allelic variants—which have a major impact on RBC folate levels—may account for a significant degree of familial aggregation in at least a subset of families.

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References

Aalen OO (1991) Modelling the influence of risk factors on familial aggregation of disease. Biometrics 47:933–945

Boushey CJ, Beresford SSA, Omenn GS, Motulsky AG (1995) A quantitative assessment of plasma homocysteine as a risk factor for vascular disease: probable benefits of increasing folic acid intake. JAMA 274:1049–1057

Butterworth CE (1991) Folate deficiency in cancer. In: Bendich A, Butterworth CE (eds) Micronutrients in health and disease prevention. Marcel Dekker, New York, pp 165–183

Cowchock S, Ainbender E, Prescott G, Crandall B, Lau L, Heller R, Muir WA, et al (1980) The recurrence risk for neural tube defects in the United States: a collaborative study. Am J Med Genet 5:309–314

Cragan JD, Roberts HE, Edmonds LD, Khoury MJ, Kirby RS, Shaw GM, Velie EM, et al (1995) Surveillance for anencephaly and spina bifida and the impact of prenatal diagnosis—United States, 1985–1994. MMWR 44:1–13

Czeizel AE (1996) Reduction of urinary tract and cardiovascular defects by periconceptional vitamin supplementation. Am J Med Genet 62:179–183

Czeizel AE, Dudas I (1992) Prevention of first occurrence of neural-tube defects by periconceptional vitamin supplementation. N Engl J Med 327:1832–1835

Daly LE, Kirke PN, Molloy A, Weir DG, Scott JM (1995) Folate levels and neural tube defects: implications for prevention. JAMA 274:1698–1702

Davis RE (1986) Clinical chemistry of folic acid. Adv Clin Chem 25:233–294

Hopper JL, Carlin JB (1992) Familial aggregation of a disease consequent upon correlation between relatives in a risk factor measured on a continuous scale. Am J Epidemiol 136: 1138–1147

Lange K, Weeks D, Boehnke M (1988) Programs for pedigree analysis: MENDEL, FISHER and dGENE. Genet Epidemiol 55:471–472

Leck I (1977) Folates and the fetus. Lancet 1:1099–1100

Li DK, Daling JR, Mueller BA, Hickok DE, Fantel AG, Weiss NS (1995) Periconceptional multivitamin use in relation to the risk of congenital urinary tract anomalies. Epidemiology 6:212–218

Mitchell LE, Duffy DL, Martin NG (1994) Evidence for familial effects on variation in red blood cell folate: implications for the familial aggregation of neural tube defects. Am J Hum Genet Suppl 55:A47

MRC Vitamin Study Research Group (1991) Prevention of neural tube defects: results of the Medical Research Council vitamin study. Lancet 338:131–137

Rosenblatt DS (1989) Inherited disorders of folate metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic basis of inherited disease, 6th ed. McGraw-Hill, New York, pp 2049–2064

SAS Institute (1991) The SAS system, version 6.10. SAS Institute, Cary NC

Scott JM, Weir DG, Molloy A, McPartlin J, Daly L, Kirke P (1994) Folic acid metabolism and mechanisms of neural tube defects. Ciba Foundation Symposium. Vol 181. John Wiley, Chichester, England

Shaw GM, Lammer EJ, Wasserman CR, O'Malley CD, Tolarova MM (1995) Risk of orofacial clefts in women using multivitamins containing folic acid periconceptionally. Lancet 346:393–396

Shaw GM, Wasserman CR, O'Malley CD (1994) Periconceptional vitamin use and reduced risk for conotruncal and limb defects in California. Teratology 49:372

Treloar SA, Martin NG, Dennerstein L, Raphael B, Heath AC (1992) Pathways to hysterectomy: insights from longitudinal twin research. Am J Obstet Gynecol 167:82–88

Wald N (1993) Folic acid and the prevention of neural tube defects. Ann NY Acad Sci 678:112–129

Zackai EM, Spielman RS, Mellman RJ, Ames M, Bodurtha J (1978) The risk of neural tube defects to first cousins of affected individuals. In: Crandall BF, Brazier MZ (eds) The prevention of neural tube defects: the role of alpha-fetoprotein. Academic Press, New York, pp 99–102