Maternal serum folate and vitamin B₁₂ concentrations in pregnancies associated with neural tube defects

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SUMMARY Serum folate and vitamin B₁₂ concentrations in early pregnancy were compared for 32 mothers with pregnancies affected by neural tube defects and 395 randomly selected pregnant control women from the same maternity hospitals. No significant differences were found between the affected mothers and the controls in the median values and frequency distributions of either vitamin. Sixteen of the samples from mothers whose infants had neural tube defects were taken between 9 and 13 weeks' gestation and 11 of these had both serum folate and vitamin B₁₂ concentrations within the normal ranges for our laboratory. These findings are discussed in relation to the concept of folate deficiency as a major factor in the aetiology of neural tube defects.

There is currently much interest in the possible role of vitamin deficiencies in the aetiology of neural tube defects. This follows two recent studies which indicated that periconceptional supplementation with a multivitamin, multimineral preparation containing folic acid¹ and with folic acid alone² may reduce the risk of recurrence of neural tube defects. Because of difficulties in interpreting the findings of these intervention studies a randomised clinical trial began in the Dublin maternity hospitals in 1981, and a similar trial is now being carried out by the Medical Research Council of the United Kingdom to determine whether periconceptional supplementation with multivitamins and multiminerals or folic acid reduces the risk of recurrence of this malformation. These intervention studies^{1 2} followed an earlier report of an association between the birth of a baby with a neural tube defect and low concentrations of some vitamins, notably folic acid, vitamin C,³ and vitamin B₁₂⁴ in the mother's blood in early pregnancy. These findings, however, were based on only six cases of neural tube defect.

We describe a study using serum samples collected from nearly 18 000 women in early pregnancy as a routine rubella antibody screen. Pregnancies resulting in infants with a neural tube defect were identified and maternal serum folate and vitamin B₁₂ concentrations were compared between affected pregnancies and randomly selected controls.

Materials and methods

When a mother first attends the antenatal clinic in the Dublin maternity hospitals a serum sample for rubella antibody screening is routinely taken unless there is previous serological evidence that she is already immune. Serum samples are separated after collection of blood specimens in each of the hospitals and forwarded to the Virus Reference Laboratory, University College Dublin where they are consecutively numbered on arrival and either tested for rubella antibody within 24 hours of the time of taking the sample or stored at –20°C until tested. At no stage does any sample remain at room temperature for more than 24 hours. After analysis any remaining serum is stored at –20°C for 10 months. For this study all serum samples (17 861) from three of the four major Dublin maternity hospitals which were tested for rubella antibody during a defined two year period (Table 1) were made available by the Virus Reference Laboratory. These samples related to births occurring during the period 1981 to mid-1983 approximately. As the case finding and sample analysis were carried out during the first half of 1983 it was not possible for us to obtain complete lists of births of infants with neural tube defects during this period and so we confined our search for affected infants to those born during 1981 and 1982. In early 1983 the names and addresses of 116

Table 1 Details of sample collection of mothers whose pregnancies were affected by neural tube defects (NTD) and controls by hospital

Hospital	Sample collection period	Total no of specimens in sampling period	No of NTD specimens obtained	No of controls sampled
1	26 Sep 1980–29 Oct 1982	8,233	16	182
2	23 Sep 1980–22 Sep 1982	8,617	14	193
3	1 Aug 1981–26 Mar 1982	1,011	2	20
All hospitals		17,861	32	395

mothers who had delivered babies with neural tube defects in the three study hospitals during 1981–2 were available to us. Only two of 20 affected infants were traced in the rubella sample lists for hospital 3 because the lists were incomplete and spanned a period of only eight months (Table 1). Of the 96 mothers whose babies had neural tube defect in hospitals 1 and 2, 45 did not have a rubella sample taken during the study pregnancy because serological evidence was available of immunity in a previous pregnancy; 41 mothers had a rubella sample taken but in four this was taken before the sample collection period for our study. In the initial search for samples it seemed that the remaining 10 mothers had not had a rubella sample taken because they were not traced in the rubella lists. A subsequent detailed recheck showed that all 10 had had a rubella sample and were missed because of linkage failures between the two lists, usually due to changes or inaccuracies in names or addresses, or both. These mothers were not included in the serum analysis because a year had elapsed since the analysis of the main study samples. Serum samples from 39 mothers (37 from hospitals 1 and 2 and two from hospital 3) whose pregnancies had resulted in infants with a neural tube defect were identified for assay but on further examination two of the 18 in hospital 1 and five of the 19 in hospital 2 were excluded for the following reasons: incorrect diagnosis (three); rubella sample not taken during the affected pregnancy (two); and insufficient serum for

analysis (two). Thus, 32 specimens obtained from the rubella serum banks were suitable for analysis and these related to 16 cases of anencephalus, 15 cases of spina bifida, and one case of encephalo-coele. A control group was selected by taking a random sample of all rubella serum samples for the same time period for each hospital using the sequential sample numbering system of the Virus Reference Laboratory. Approximately 10 controls were selected for each of the 39 infants having a neural tube defect for which a serum sample was originally traced (Table 1). A check of the controls against the lists of neural tube defect cases for the period 1981 to mid-1983 confirmed that none were included in the controls. During relocation and selection for the study all samples were maintained at -20°C and were thawed only at the time of analysis. Ascorbic acid was not added to specimens because of its adverse effects on vitamin B₁₂ estimation. We were aware of the possibility that some folate might have been lost from serum during the handling necessary for rubella analysis and subsequent long term storage, since this has been reported to occur in samples not containing ascorbic acid,⁵ however, such deterioration would be likely to occur equally among control and neural tube defect samples. Both folate and vitamin B₁₂ concentrations in serum samples were estimated using established microbiological assays based on the growth of chloramphenicol resistant *Lactobacillus casei*⁶ and *L leichmanii*⁷ for the respective vitamins.

Table 2 Serum folate and vitamin B₁₂ concentrations for control and study groups for combined hospitals

	Group	No of cases	Range	Median	First quartile	Third quartile
Serum folate (ng/ml)	{ Control	384	0.6–20	3.4	2.2	6.8
	{ Study	32	1.0–20	3.4	2.2	6.4
Serum vitamin B ₁₂ (pg/ml)	{ Control	363	75–1000	277	222	360
	{ Study	28	175–600	297	222	390

Results

An analysis of the combined results from all hospitals is presented in Table 2. The range of values incorporates the full sensitivity range of the assays. The maximum values given (20 ng/ml for folates and 1000 pg/ml for vitamin B₁₂) are the upper limits of sensitivity of the assay systems using standard amounts of serum. No attempt was made to dilute serum to obtain values above these upper limits. As the presence of a few very high values resulted in a skewed distribution, the median (rather than the mean) and the first and third quartile values are given. There were no significant differences between the study group and the control group. The median and the first and third quartile values of both serum folate and serum vitamin B₁₂ were similar for the neural tube defect group and the controls (Table 2).

To analyse the frequency distributions, the data were subclassified as follows: 0 to 2 ng/ml; 2.01 to 2.7 ng/ml; and 2.71 to 20 ng/ml—based on deficiency, possible deficiency, and sufficiency ranges for serum folate in our laboratory, and 0 to 150 pg/ml; 151 to 250 pg/ml; and 251 to 1000 pg/ml based on a similar classification for serum vitamin B₁₂ (Table 3). Using the χ^2 test, there were no significant differences between the control and the study groups in serum folate or serum vitamin B₁₂ concentrations although the vitamin B₁₂ values tended to be higher in the study group (Table 3). None of the study group and only six of the controls had a serum folate value less than 1 ng/ml. Some 21.9% of samples from the neural tube defect group and 22.8% of control samples were deficient in either folate or vitamin B₁₂. Some 43.8% of study group samples, however, and 35.7% of controls had sufficient serum concentrations of both folate and vitamin B₁₂ (serum folate greater than 2.7 ng/ml and

Figure Serum folate concentrations in 32 mothers whose pregnancies were affected by neural tube defect by week of gestation.

Figures refer to case numbers mentioned in text.

serum vitamin B₁₂ greater than 250 pg/ml). The data were further analysed for each hospital separately (not shown) and again no significant differences were found between the groups.

Serum folate values are plotted for the study group by week of gestation at the time of the blood test (Figure). It can be seen that 16 samples were taken between 9 and 13 weeks' gestation, and 14 were within the sufficiency range of our laboratory (greater than 2.7 ng/ml). In three cases (17, 29, and 30) vitamin B₁₂ values were less than 250 pg/ml. Thus, 11 samples taken within the first 13 weeks of pregnancy had both serum folate and vitamin B₁₂ values within normal ranges.

The data for the study group mothers were also examined with respect to the type of neural tube defect, since an earlier report⁴ indicated that low serum vitamin B₁₂ concentrations might be associated with anencephaly. The median, first, and third quartiles for serum vitamin B₁₂ values of 14 mothers whose infants had spina bifida were 300 pg/ml, 225 pg/ml, and 395 pg/ml respectively. Corresponding values for 13 mothers whose infants had anencephaly were 300 pg/ml, 215 pg/ml, and 410 pg/ml. One mother whose infant had encephalo-

Table 3 Distribution of serum folate and vitamin B₁₂ concentrations in study and control groups

	Control group No (%)	Study group No (%)
Serum folate		
0-2.0 ng/ml	74 (19.3)	7 (21.9)
2.01-2.7 ng/ml	65 (16.9)	4 (12.5)
2.71-highest	245 (63.8)	21 (65.6)
Total	384 (100)	32 (100)
Serum vitamin B₁₂		
0-150 pg/ml	23 (6.3)	0 (0)
151-250 pg/ml	118 (32.5)	10 (35.7)
251-highest	222 (61.2)	18 (64.3)
Total	363 (100)	28 (100)

χ^2 for folate=0.47; $P>0.70$; not significant.

χ^2 for vitamin B₁₂=1.90; $P>0.25$; not significant.

coele had a serum vitamin B₁₂ value of 212 pg/ml. No significant differences were found between these groups. Similarly no differences were found in the serum folate values in relation to the type of neural tube defect.

Discussion

It has been suggested that maternal nutritional status, particularly with reference to folic acid, plays a part in the aetiology of neural tube defects.¹⁻³ If deficiency of a nutrient is involved it might be reflected in a low serum status for that particular nutrient before or during the affected pregnancy. The relatively low prevalence at birth of the defect, however, makes a prospective study in this area a daunting task. As a result prospective studies that have examined the role of nutritional factors have been based on very small numbers of pregnancies affected by neural tube defects.^{2,3,8,9}

Because our study was based on serum samples collected routinely for rubella antibody screening between 1980 and 1982, we were able to trace samples for 32 pregnancies affected by neural tube defects. We decided as a priority to estimate serum values of two previously implicated vitamins, folic acid^{2,3} and vitamin B₁₂.⁴ Since the serial identification numbers of the serum banks used in this study are based only on the order of presentation of a mother for rubella testing it was considered that a random sample of these specimens would provide an unbiased control group. No significant differences were found in the serum folate or vitamin B₁₂ concentrations between the mothers of infants with neural tube defects and the controls.

The relatively high prevalence of folate deficiency in the total study population may be due to several factors. As already mentioned some folate may have been lost from serum stored without ascorbic acid. The main objective of this study, however, was a comparison of sera from mothers with neural tube defect pregnancies with controls rather than an evaluation of absolute folate values in serum. The method of selection of controls from the same time period makes it likely that deterioration, if it occurred, would be similar in both groups. Another possibility may be the increased prevalence of folate deficiency among pregnant women in general. Hall⁸ reported that six per cent of pregnant women attending their first antenatal clinic had serum folate concentrations less than 2.9 ng/ml, but the deficiency range was not given. Serum folate values fall during pregnancy¹⁰ with the most rapid decline occurring between 12 and 20 weeks' gestation,¹¹ and low serum folates have been reported in up to 50% of women in the late stages of pregnancy.¹⁰ Serum

vitamin B₁₂ values also decrease throughout pregnancy,^{10,12} and values of less than 150 pg/ml have been recorded in 14% to 28% of mothers by the third trimester.¹⁰ The incidence of folate deficiency in pregnancy may be higher in Dublin than elsewhere, but a comparison between studies is difficult because of laboratory differences in measuring folates.

Blood concentrations of various vitamins have been measured in women during pregnancies affected by neural tube defects.^{2-4,13} Smithells and his colleagues³ found significantly lower mean red cell folates during the first trimester in six mothers with affected pregnancies compared with a control group. The mean serum folate concentration in the study group was also lower, but the difference was not statistically significant. In a study by Laurence *et al*,² mothers who had previously had an infant affected by neural tube defect were randomised to receive either folic acid or a placebo. The mean red cell folate concentration during the second month of pregnancy of six women with a recurrence of neural tube defect was lower than the mean of a placebo group, but not significantly so. Low red cell folate independent of low serum folate might imply either vitamin B₁₂ deficiency or some deficiency in cellular uptake mechanisms. Indeed, vitamin B₁₂ values were low or marginally low in three of six mothers of infants suffering a neural tube defect in the Smithells study⁴ and in five of the six samples in the Laurence study.¹³ The sixth sample in the Laurence study with normal concentrations of vitamin B₁₂ had a very low red cell folate value, but also had the lowest serum folate of the group. In the Smithells study⁴ the serum vitamin B₁₂ concentrations of the mothers of three anencephalic babies were very low when compared with two cases of spina bifida and with a control group. The associations between low blood folate and vitamin B₁₂ concentrations and neural tube defects reported in these studies are based on small numbers of cases and are, therefore, questionable. In an unselected series Hall^{8,9} found no difference in the mean serum folate value at the first antenatal clinic between nine mothers whose pregnancies resulted in babies with a neural tube defect and the total study population of 2949 mothers. In our study, 32 samples from mothers with affected babies did not differ in respect of serum folate and vitamin B₁₂ values from a group of randomly selected controls for the same time period. Our results therefore are in agreement with those of Smithells *et al*³ and Hall⁹ in respect of serum folate values. A possible association between vitamin B₁₂ deficiency and anencephaly⁴ was not confirmed in this study. Assuming vitamin B₁₂ sufficiency, red cell folate rather than serum folate taken during

early pregnancy, may be a better reflection of folate status at the time of neural tube closure. Unfortunately, an estimation of red cell folates could not be done in this study. Nevertheless, the presence of normal serum values of folate and vitamin B₁₂ in so many of these study group samples so soon after defective closure of the neural tube suggests that this event can take place in the presence of normal circulating values of these vitamins.

If folate deficiency is an important factor in the aetiology of neural tube defects how can our findings be explained? We cannot assume that the 32 mothers of babies with this disorder included in our study are representative of the total group of neural tube defect pregnancies occurring in the hospitals during the study period. The mothers with affected infants not included might have had lower serum vitamin values than the study mothers but this cannot be ascertained. It is theoretically possible that a comparison between all of the mothers with babies affected by neural tube defects and the control group might have shown lower vitamin values in the former group.

The possibility that the study mothers may have been on folate supplements before the date of the rubella sample was considered. Although we presumed that this might have been the reason for the high serum folate concentrations of cases 3, 9, 21, and 25 in the Figure, examination of the hospital charts showed evidence of supplementation in only one case (25). Iron and vitamin supplementation is usually started at the first antenatal clinic and the hospital charts confirmed that the rubella test had been taken at this visit for all but two of these mothers. Should supplementation before the test be a confounding factor we cannot see that it would have been more common among the study mothers than the controls.

Alterations of dietary habits to high or low folate intake are reflected initially in changing serum folate values.^{14 15} A selective improvement in diet, however, before and after conception by the study mothers compared with controls seems unlikely. Day to day variations in serum folate may occur in a particular subject. On a reasonably similar diet over a period of time, however, the serum folate concentrations of an individual are unlikely to fluctuate dramatically between deficiency and sufficiency ranges (Ek, J, personal communication). It is unlikely, therefore, that all or most of the study mothers with normal serum folate concentrations at 9 to 13 weeks' gestation could have had deficient values at 5 weeks' gestation.

This study raises questions about the role of folate deficiency in the aetiology of neural tube defects. Although the findings must be interpreted with

caution they support the view that folate deficiency may be neither the main nor the sole cause of neural tube defects,¹⁶ and they are also consistent with the possibility that folate deficiency may not play a part in the aetiology of these malformations.^{8 9} Deficient red cell folate concentrations in association with normal serum folate values imply diminished uptake of folate into tissues, either due to vitamin B₁₂ deficiency or to some other uptake or transport process. Our finding of similar serum concentrations of vitamin B₁₂ among the study mothers and controls suggests that if folate is involved the association may not be deficiency in the conventional sense, and maternal blood values of the vitamin may not reflect availability to the fetus. Further basic experimental work on maternal transfer and fetal uptake and transport of these vitamins is required. In addition, our results emphasise the importance of the randomised trials now underway in Dublin and in Britain to establish whether supplementation with folate or a multivitamin preparation can prevent the recurrence of neural tube defects.

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