ORIGINAL ARTICLE

# Autoantibodies against Folate Receptors in Women with a Pregnancy Complicated by a Neural-Tube Defect

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## ABSTRACT

### BACKGROUND

In the absence of clinical folate deficiency, periconceptional supplementation with folic acid reduces a woman's risk of having an infant with a neural-tube defect. Since antiserum to folate receptors induces embryo resorption and malformations in rats, we hypothesized that autoantibodies against folate receptors in women may be associated with pregnancy complicated by a neural-tube defect.

### METHODS

Serum from 12 women who were or had been pregnant with a fetus with a neural-tube defect and from 24 control women (20 with current or prior normal pregnancies and 4 who were nulligravid) was analyzed for autoantibodies by incubation with human placental folate receptors radiolabeled with [$^3$H]folic acid. The properties of these autoantibodies were characterized by incubating serum and the autoantibodies isolated from serum with placental membranes, ED27 cells, and KB cells, which express the folate receptors.

### RESULTS

Serum from 9 of 12 women with a current or previous affected pregnancy (index subjects) and 2 of 20 control subjects contained autoantibodies against folate receptors ($P<0.001$). The autoantibodies blocked the binding of [$^3$H]folic acid to folate receptors on placental membranes and on ED27 and KB cells incubated at 4°C and blocked the uptake of [$^3$H]folic acid by KB cells when incubated at 37°C.

### CONCLUSIONS

Serum from women with a pregnancy complicated by a neural-tube defect contains autoantibodies that bind to folate receptors and can block the cellular uptake of folate. Further study is warranted to assess whether the observed association between maternal autoantibodies against folate receptors and neural-tube defects reflects a causal relation.

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Neural-tube defects, which include spina bifida, anencephaly, craniorachischisis, and encephalocele, occur in approximately 1 per 1000 births in the United States, and women who have one fetus with this complication are at increased risk in subsequent pregnancies.[1] Although periconceptional folic acid supplementation reduces the occurrence and recurrence of neural-tube defects by approximately 70 percent,[2,3] most women who are pregnant with a fetus with this complication do not have clinical folate deficiency.[4] Though some polymorphisms for folate-pathway enzymes have been identified,[5] they cannot account for the 70 percent decrease in the incidence of this birth defect with folate supplementation.

Studies in animals have suggested the importance of folate receptors in embryogenesis.[6] Inactivation of both alleles encoding the mouse homologue of the human folate receptor $\alpha$ gene was uniformly fatal in embryos with neural-tube defects.[7,8] Folinic acid given to the pregnant dams resulted in normal development in 80 percent of the embryos that lacked the folate receptor $\alpha$ gene in both alleles.[8] Despite considerable research, no specific polymorphisms or mutations of the human folate receptor gene have been identified that might explain the reduction in the incidence of neural-tube defects with folic acid supplementation.[9]

Our observation that the administration of an antiserum to folate receptors[10] to pregnant rats resulted in the resorption of or multiple developmental abnormalities in embryos[11] prompted us to speculate that an autoantibody against folate receptors in women could induce similar embryonic and fetal abnormalities. Therefore, we developed an assay to measure these autoantibodies in the serum of women with a current or prior pregnancy complicated by a neural-tube defect and that of women without such a history.

## METHODS

### SUBJECTS

Between April 2002 and August 2003, all 12 women at our institution with a current or prior pregnancy complicated by a neural-tube defect who consented to participate in this study were enrolled and had their serum analyzed for autoantibodies against the folate receptor. The women were attending the obstetrical clinic or were the mothers of children with neural-tube defects who were being followed in the pediatric neurology clinic. Eligible women were between the ages of 18 and 40 years at the time of the affected pregnancy.

Initially, we enrolled 12 women with a current or previous affected pregnancy (index subjects) and 18 control subjects who had never had a pregnancy involving a neural-tube defect, 4 of whom were nulligravid. An initial analysis comparing the frequency of the autoantibodies in the group of index subjects and these 18 controls showed a significant difference ($P<0.001$).

Subsequently, we decided that women who were pregnant at the time of testing with a fetus that did not have a neural-tube defect would be more appropriate additional controls. Accordingly, the first six women who met this criterion and provided informed consent were also enrolled in the study. Thus, there were 24 controls; 8 were pregnant with an unaffected fetus, 12 had given birth to one or more unaffected infants, and 4 were nulligravid. Because pregnancy was a criterion for study entry, the four nulligravid control women who were initially tested for the autoantibody were excluded from the statistical analysis. Although the number of subjects in each group was not prespecified, every woman who consented to participate was included in the study. The institutional review board approved the study, and written informed consent was obtained from all subjects.

### PREPARATION OF [$^3$H]FOLIC ACID–LABELED PURIFIED FOLATE RECEPTORS

Folate receptors from human placental membranes were solubilized at 37°C for two hours in 0.01 M sodium phosphate buffer, pH 7.4, containing 1 percent Triton X-100, and purified with Sepharose 4B to which folic acid was covalently coupled.[12] The matrix was washed with the phosphate buffer, and the receptors were eluted from the matrix with 0.1 M glycine–hydrochloric acid, pH 3.5, containing 0.1 percent Triton X-100. The pH of the eluate was raised to 7.4 by the addition of 0.2 M sodium barbiturate, and the receptors were quantified on the basis of the level of binding of [$^3$H]folic acid (specific activity, 31 Ci per millimole; Moravek), which binds at a 1:1 molar ratio.

Radiolabeled receptors were prepared by incubating the purified apo-folate receptors with a 20 percent excess of [$^3$H]folic acid for 30 minutes at 4°C. At a pH between 7 and 9, there is virtually no dissociation of folic acid from the receptors incubated overnight at 4°C. The addition of a 10-fold greater concentration of the folate receptors, saturated with



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nonradioactive folic acid, confirmed the specificity of the folate receptor–autoantibody complex.

### ASSAY OF SERUM FOR THE AUTOANTIBODY AGAINST THE FOLATE RECEPTORS

For the assay for the autoantibody against folate receptors, 40 μl of serum was added to 200 μl of 0.1 M glycine buffer, pH 3.0, incubated for 10 minutes at 25°C, and then added to a pellet of dextran-coated charcoal to adsorb free folate and folate that dissociated from soluble receptors. At this pH, soluble receptors will also dissociate from autoantibodies in the serum. The charcoal was pelleted, and the supernatant fraction was added to 200 μl of 0.2 M sodium barbiturate, pH 8.9, containing phenylmethylsulfonyl fluoride, EDTA, 0.1 percent Triton X-100, and the [$^3$H]folic acid–folate receptor preparation. Another aliquot of serum was added to a mixture of the [$^3$H]folic acid–folate receptors containing a 10-fold excess of the receptors saturated with nonradioactive folic acid.

The volume of each reaction was increased to 600 μl by the addition of glycine–sodium barbiturate buffer (final pH, 8.6), incubated overnight at 4°C, and 600 μl of a 10 percent suspension of staphylococcal protein A membranes (Pansorbin, Calbiochem) was added. The incubation continued for 15 minutes; the membranes were then pelleted and washed, and the level of radioactivity was determined. The level of background radioactivity measured in duplicate reactions that lacked serum was subtracted from the test serum samples.

### AUTOANTIBODIES AND THE BINDING OF [$^3$H]FOLIC ACID TO FOLATE RECEPTORS

Membranes were prepared from human placenta homogenized in 0.01 M sodium phosphate buffer, then pelleted, and washed. The membranes were suspended in 0.1 M acetic acid to dissociate endogenous folate from the receptors, washed twice in this solution, and resuspended in 0.01 M sodium phosphate buffer, pH 7.4. The binding of [$^3$H]folic acid was used to determine the quantity of the apo-folate receptors.

The effect of the autoantibodies on the binding of [$^3$H]folic acid to the receptors was determined by incubating 200 μl of a 1 percent suspension of the membranes with charcoal-treated serum overnight at 4°C. The membranes were washed with cold phosphate-buffered saline, incubated for 30 minutes at 4°C with [$^3$H]folic acid (125 pg) in 1 ml of phosphate-buffered saline, washed with cold buffer, and solubilized with 1 N sodium hydroxide, and the radioactivity was determined.

ED27 cells, a human-placenta cell line,[13] and KB cells, a human epidermoid carcinoma-cell line,[14] were each plated in triplicate at a density of 20,000 cells in 1.83-cm$^2$ wells containing folate-deficient Dulbecco's minimum essential medium with 10 percent fetal-calf serum and the test serum (pretreated with charcoal to remove free endogenous folate) and incubated overnight at 37°C. The cells were washed with cold Hanks' balanced salt solution (HBSS) and incubated at 4°C for 30 minutes with [$^3$H]folic acid (125 pg) in 1 ml of HBSS. The samples were washed with cold HBSS, and the cell-associated radioactivity was determined.

To obtain the apparent association constant ($K_a$) for the binding of the autoantibodies to the folate receptors, the charcoal-treated serum was incubated overnight at 4°C with placental membranes containing the apo-folate receptors, followed by incubation with [$^3$H]folic acid for 30 minutes. The free ligand was removed by pelleting the membranes, and the radioactivity of the membrane-bound [$^3$H]folic acid was determined. This value represented the apo-folate receptors that were not blocked by the autoantibodies. Scatchard analysis[15] was used to compute the apparent $K_a$.

### AUTOANTIBODIES AND THE CELLULAR UPTAKE OF [$^3$H]FOLIC ACID

Ten milliliters of serum from Index Subject 8 and from Control Subject 14 (see Supplementary Appendixes 1 and 2, available with the full text of this article at www.nejm.org) was incubated overnight at 4°C with 1 ml of packed placental membranes to bind the autoantibodies to the folate receptors. The samples were washed with cold HBSS to remove unbound serum proteins, and autoantibodies bound to the folate receptors were eluted with 0.1 M acetic acid containing 0.1 percent bovine serum albumin. The eluate was dialyzed against HBSS at 4°C overnight and concentrated by vacuum dialysis.

KB cells (20,000) plated in duplicate in 3.5-cm$^2$ culture dishes were incubated overnight at 37°C with the isolated autoantibodies, the similarly treated control serum, or medium alone. The cells were washed with HBSS, medium containing [$^3$H]folic acid (125 pg) was added to the wells, and the incubation was continued for 15, 30, and 60 minutes at 37°C, with duplicate sets incubated at 4°C. The cells were washed again with HBSS at 4°C and lysed with 1 N sodium hydroxide, and the radio-



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activity was determined. The difference in radioactivity between the cells incubated at 4°C and those incubated at 37°C represents the cellular uptake of [$^3$H]folic acid.

The analysis of serum from index and control subjects for other autoantibodies was performed by Universal Diagnostic Laboratory with use of enzyme-linked immunosorbent assays (anti-DNA antibodies from Wampole Laboratories; anticardiolipin antibodies from DiaSorin; antithyroid antibodies from Diagnostic Products).

### STATISTICAL ANALYSIS

The percentage of women with autoantibodies to the folate receptor in the two groups was compared with the use of Fisher's exact test. Mean binding levels of the [$^3$H]folic acid–folate receptor complex in serum were compared between groups with the use of Student's t-test; Satterwaite's correction of the degrees of freedom was applied because of the unequal variances. Clinical variables in the two groups were compared with the use of the Wilcoxon two-sample test and Fisher's exact test. Two-tailed tests were used in all cases; P values of 0.05 or less were considered to indicate statistical significance. SAS software (SAS Institute) was used.

## RESULTS

Table 1 shows the demographic and clinical characteristics of the 12 index subjects and the 20 control subjects who had been or were pregnant. There were no significant differences between the groups. Other clinical characteristics of the index and control subjects (including the four nulligravid controls who were not included in the statistical analyses) are provided in Supplementary Appendixes 1 and 2 (available with the full text of this article at www.nejm.org).

Autoantibodies against the folate receptors were identified in the serum of 9 of 12 index subjects (75 percent) — 2 with a current pregnancy complicated by a neural-tube defect (Subjects 4 and 5) and 7 (Subjects 1, 2, 3, 6, 7, 8, and 9) with a history of a pregnancy complicated by a neural-tube defect (Fig. 1A) — as compared with 2 of the 20 control subjects (10 percent, P<0.001) (Fig. 1B). The mean value for the [$^3$H]folic acid–labeled folate receptors bound by the autoantibodies per milliliter of serum from the index subjects (27,705±5842 dpm) was significantly greater than that for the 20 controls (3279±1285 dpm, P=0.002) or that for the 8 control subjects who were pregnant when blood was obtained (5779±2982 dpm, P<0.001).

Figure 2 shows the effect of the autoantibodies on the binding of [$^3$H]folic acid to folate receptors. Serum from Index Subjects 7 and 8 was selected on the basis of the availability of serum required for this analysis and contained autoantibodies that blocked the binding of [$^3$H]folic acid to placental membrane receptors, ED27 cells, and KB cells at 4°C, whereas serum from five control subjects (Subjects 5, 6, 10, 11, and 14), which tested negative for autoantibodies, did not. Autoantibodies isolated from the serum of Index Subject 8 blocked 95 percent of the cellular uptake of [$^3$H]folic acid by KB cells at 37°C (Fig. 3), indicating that the epitope on the receptor is either at or close to the ligand-binding site.

Figure 4 shows the binding of apo-folate receptors to the autoantibodies in the serum from three index and two control subjects; the insets show the Scatchard analysis.[15] Index Subjects 8 and 9 appeared to have a single order of autoantibody-binding sites on the receptor with similar $K_a$ values

**Table 1. Clinical Characteristics of the Subjects.***

| Characteristic | Index Subjects (N=12) | Control Subjects (N=20) | P Value |
|---|---|---|---|
| Age — yr | | | 0.06† |
| Median | 26.5 | 32 | |
| Range | 21–35 | 22–40 | |
| Gravida — no. | | | 0.97† |
| Median | 3 | 3 | |
| Range | 1–6 | 1–12 | |
| Para — no. | | | 0.25† |
| Median | 1 | 1 | |
| Range | 0–4 | 0–3 | |
| Miscarriages — no. | | | 0.13† |
| Median | 0 | 1 | |
| Range | 0–2 | 0–8 | |
| Induced abortions — no. | | | 0.37† |
| Median | 0.5 | 0 | |
| Range | 0–2 | 0–4 | |
| Medical illnesses — no. | 4 (33)‡ | 4 (20)§ | 0.43¶ |
| Racial or ethnic group — no. (%) | | | 0.55¶ |
| Hispanic | 3 (25) | 3 (15) | |
| Black | 6 (50) | 8 (40) | |
| Asian | 1 (8) | 1 (5) | |
| White | 2 (17) | 8 (40) | |

* Four nulligravid control subjects were not included in the statistical analysis.
† The P value was calculated with the use of Wilcoxon's two-sample test.
‡ The illnesses were asthma, epilepsy, diabetes, and morbid obesity.
§ The illnesses were pulmonary disorder, thyroiditis, idiopathic autoimmune thrombocytopenia, gestational diabetes, and morbid obesity.
¶ The P value was calculated with the use of Fisher's exact test.



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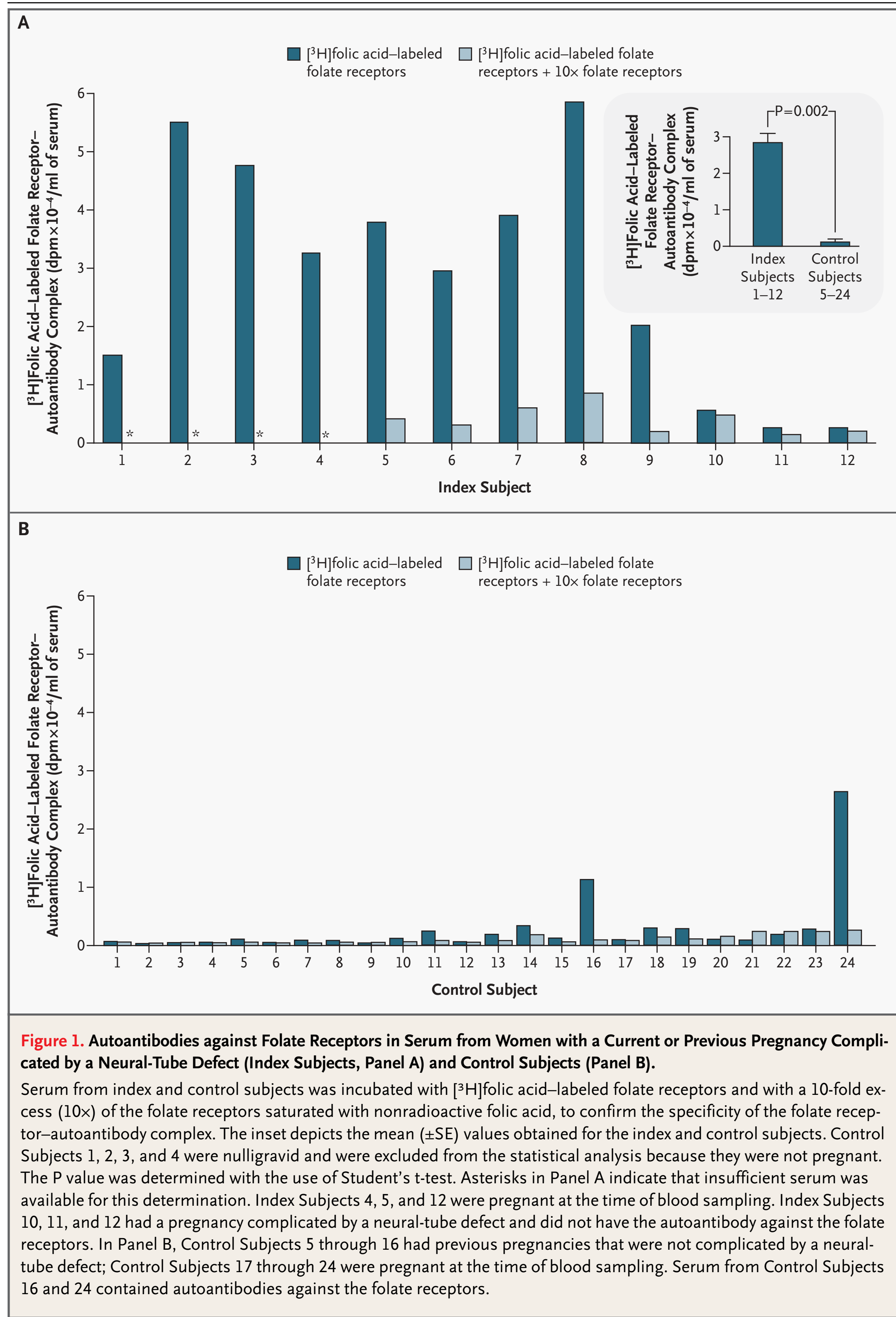


**Figure 1. Autoantibodies against Folate Receptors in Serum from Women with a Current or Previous Pregnancy Complicated by a Neural-Tube Defect (Index Subjects, Panel A) and Control Subjects (Panel B).**

Serum from index and control subjects was incubated with [$^3$H]folic acid–labeled folate receptors and with a 10-fold excess (10×) of the folate receptors saturated with nonradioactive folic acid, to confirm the specificity of the folate receptor–autoantibody complex. The inset depicts the mean (±SE) values obtained for the index and control subjects. Control Subjects 1, 2, 3, and 4 were nulligravid and were excluded from the statistical analysis because they were not pregnant. The P value was determined with the use of Student's t-test. Asterisks in Panel A indicate that insufficient serum was available for this determination. Index Subjects 4, 5, and 12 were pregnant at the time of blood sampling. Index Subjects 10, 11, and 12 had a pregnancy complicated by a neural-tube defect and did not have the autoantibody against the folate receptors. In Panel B, Control Subjects 5 through 16 had previous pregnancies that were not complicated by a neural-tube defect; Control Subjects 17 through 24 were pregnant at the time of blood sampling. Serum from Control Subjects 16 and 24 contained autoantibodies against the folate receptors.



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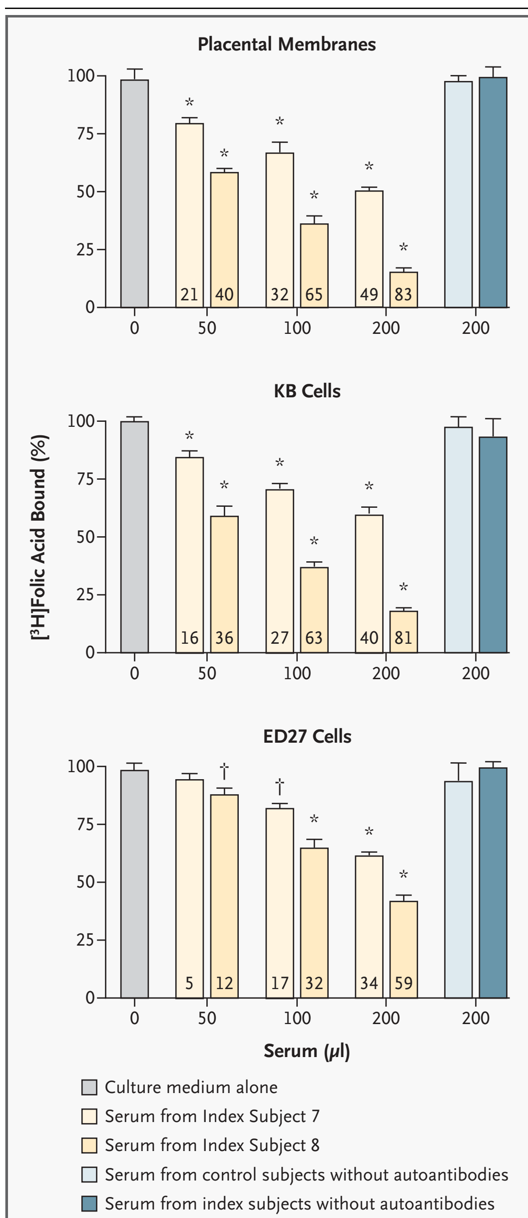


**Figure 2. Blocking of the Binding of [³H]Folic Acid to Folate Receptors on Placental Membranes, KB Cells, and ED27 Cells by Serum Containing Autoantibodies against Folate Receptors.**

The number in each bar indicates the percentage of [³H]folic acid that is blocked from binding to the apofolate receptors on the respective cell membranes by the autoantibodies at 4°C. Values are the means (±SE) of three experiments. P values are for the comparison with culture medium alone and were calculated with the use of Student's t-test. Asterisks indicate $P<0.002$, and daggers $P<0.02$.

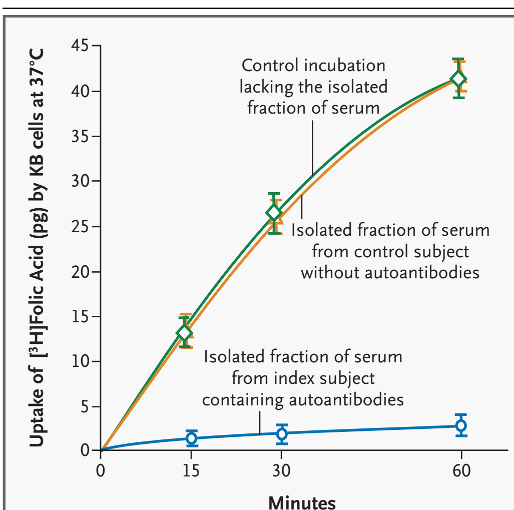


**Figure 3. Effect of Isolated Autoantibodies against Folate Receptors on the Cellular Uptake of [³H]Folic Acid by KB Cells.**

Autoantibodies against folate receptors were isolated from serum from Index Subject 8 as described in the Methods section. KB cells were incubated overnight at 37°C with this isolated fraction of serum from either an index subject or a control subject. The uptake of [³H]folic acid by the KB cells was then determined. The results of a control incubation lacking the isolated fraction of serum are also shown. The I bars represent the SE.

($2.1\times10^{10}$ and $1.8\times10^{10}$ liters per mole, respectively). Index Subject 7 appeared to have two orders of autoantibody-binding sites on the receptor ($K_a$ of $2.6\times10^{10}$ and $2.8\times10^{9}$ liters per mole). The autoantibodies in the serum from the two control subjects (Subjects 16 and 24) also appeared to have two orders of binding sites, both of which (in Subject 16) were lower than the $K_a$ for the index subjects.

The serum from the index subjects did not contain anticardiolipin, anti–single-stranded DNA, or antithyroid autoantibodies. One control subject (Subject 24) had an IgM anticardiolipin autoantibody, and another control subject (Subject 8) had an antithyroid autoantibody.

## DISCUSSION

We have identified autoantibodies against folate receptors in serum from women who have had a pregnancy complicated by a neural-tube defect. This autoantibody blocked the binding of [³H]folic acid to folate receptors on placental membranes and on



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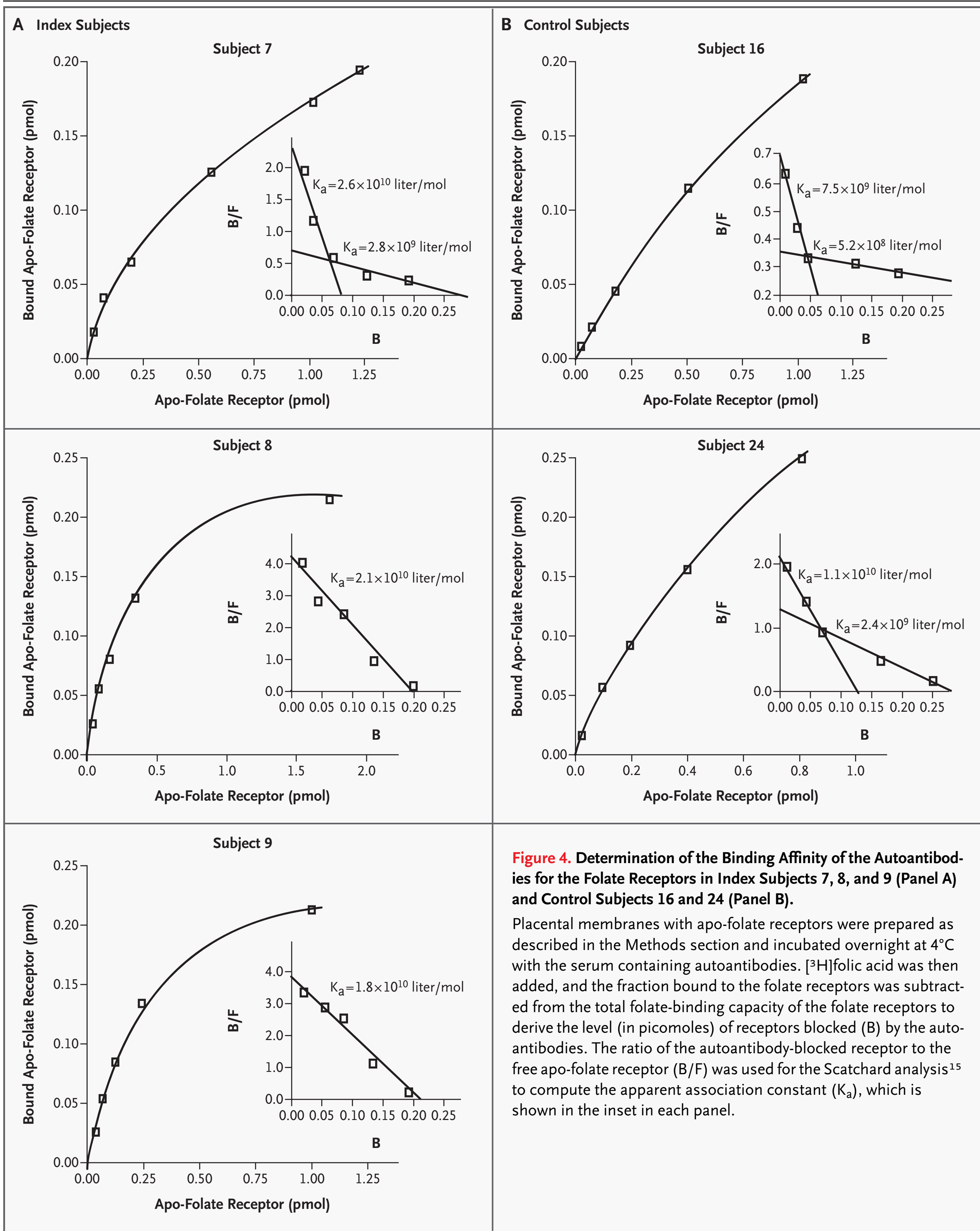


**Figure 4. Determination of the Binding Affinity of the Autoantibodies for the Folate Receptors in Index Subjects 7, 8, and 9 (Panel A) and Control Subjects 16 and 24 (Panel B).**

Placental membranes with apo-folate receptors were prepared as described in the Methods section and incubated overnight at 4°C with the serum containing autoantibodies. [$^3$H]folic acid was then added, and the fraction bound to the folate receptors was subtracted from the total folate-binding capacity of the folate receptors to derive the level (in picomoles) of receptors blocked (B) by the autoantibodies. The ratio of the autoantibody-blocked receptor to the free apo-folate receptor (B/F) was used for the Scatchard analysis[15] to compute the apparent association constant ($K_a$), which is shown in the inset in each panel.



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ED27 cells and KB cells incubated at 4°C and inhibited the uptake of folate by KB cells when incubated at 37°C. The mean apparent $K_a$ for the binding of the autoantibodies from the index subjects to the folate receptor was $2.2\times10^{10}$ liters per mole. This high affinity could explain the blocking of the binding and cellular uptake of folate.

There are multiple causes of neural-tube defects, including drugs (especially antifolate[16] and antiepileptic[17] agents), chromosomal abnormalities,[18] and environmental[19] and genetic[20] factors. Studies showing a reduction in the incidence of neural-tube defects of approximately 70 percent with periconceptional folic acid supplementation[2,3] provide evidence that supplementary folate circumvents either an impaired intracellular folate-dependent enzyme pathway or an inhibitor of the cellular uptake of folate. However, the genetic variants of folate-pathway enzymes or of folate receptors identified in women who have pregnancies complicated by a neural-tube defect do not account for the 70 percent reduction in neural-tube defects associated with folate supplementation.[21]

The occurrence of the autoantibody might explain the observed benefit of periconceptional folic acid supplementation. The autoantibody-mediated blocking of cellular folate uptake by folate receptors could be bypassed by folic acid, because it is reduced and methylated in vivo and is transported into cells by the reduced folate carrier.[22] Another potential mechanism that would be consistent with our observations is that folic acid, which has a high affinity for the receptor, may displace an autoantibody with a lower affinity for the receptor. The identification of the autoantibody against folate receptors in 75 percent of our index subjects — a rate similar to the decrease in the occurrence and recurrence of neural-tube defects of approximately 70 percent with periconceptional folic acid supplementation[2,3] — is also consistent with the possibility that folate-responsive neural-tube defects may be due to impaired folate uptake caused by autoantibodies against the folate receptors. However, the results of our cross-sectional study cannot be used to establish a cause-and-effect relation between autoantibodies against the folate receptor and neural-tube defects.

The mechanism by which folate receptors might become self antigens is not known. Since the risk of neural-tube defects may increase after abortions[23,24] or miscarriages,[25] autoimmunity may be induced by epitopes of the folate receptors exposed as a result of injury and proteolysis of the reproductive tissues, which together with host genetic factors, may trigger the generation of autoantibodies. In our study, 7 of the 12 index subjects had previously had a first pregnancy complicated by a neural-tube defect, suggesting that the autoantibodies against the folate receptors were generated before pregnancy. Their appearance could be the consequence of an unrecognized earlier spontaneous abortion, which occurs with 30 percent of implantations in women with normal fertility.[26]

Some limitations of our study should be noted. Because it was designed as a pilot study, the sample size is small, and index subjects and controls were not recruited in a systematic manner. However, selection bias is unlikely. Index and control subjects were recruited without knowledge of whether they had autoantibodies against folate receptors or other autoantibodies, and they had similar demographic and clinical characteristics. Every woman who consented to participate in this pilot study was included. We do not have longitudinal serum samples, and thus we cannot assess whether autoantibodies were present before the pregnancy involving a neural-tube defect.

In summary, we have identified an autoantibody against the folate-receptor membrane protein in women who have had a pregnancy complicated by a neural-tube defect. Additional studies are needed to establish the frequency of folate-receptor autoantibodies in women whose pregnancies are complicated by this birth defect and to determine whether these autoantibodies are pathogenic.

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