Autoantibodies to Folate Receptors in the Cerebral Folate Deficiency Syndrome

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In infantile-onset cerebral folate deficiency, 5-methyltetrahydrofolate (5MTHF) levels in the cerebrospinal fluid are low, but folate levels in the serum and erythrocytes are normal. We examined serum specimens from 28 children with cerebral folate deficiency, 5 of their mothers, 28 age-matched control subjects, and 41 patients with an unrelated neurologic disorder. Serum from 25 of the 28 patients and 0 of 28 control subjects contained high-affinity blocking autoantibodies against membrane-bound folate receptors that are present on the choroid plexus. Oral folinic acid normalized 5MTHF levels in the cerebrospinal fluid and led to clinical improvement. Cerebral folate deficiency is a disorder in which autoantibodies can prevent the transfer of folate from the plasma to the cerebrospinal fluid.
neuronal axons and dendrites. This carrier has a substrate specificity different from that of the GPI-anchored folate receptors and a lower affinity for folates.

We have considered the possibility that the low 5MTHF level in the cerebrospinal fluid of patients with cerebral folate deficiency is a consequence of impaired transport across the blood–brain and blood–cerebrospinal fluid barriers. However, we have not found abnormalities in genes encoding these receptors in cerebral folate deficiency, and the sporadic incidence of the disorder in most families further reduces the likelihood of a genetic basis for the syndrome. An alternative possibility is that impaired folate transport across the blood–cerebrospinal fluid barrier is caused by circulating autoantibodies that block the binding of folate to the GPI-anchored folate receptors. Such autoantibodies have been found in association with neural-tube defects.

METHODS

STUDY DESIGN
Between August 2003 and April 2004, serum specimens from 28 patients who had received a diagnosis of idiopathic cerebral folate deficiency were tested for the presence of autoantibodies to folate receptors. Serum specimens from 28 age-matched normal control subjects, 41 subjects with central nervous system disease unrelated to cerebral folate deficiency, and 5 mothers of patients with cerebral folate deficiency were also tested. All the subjects or their guardians provided written informed consent for participation after the study had been approved by the University Hospital Aachen ethics committee.

CHARACTERISTICS OF THE PATIENTS
The 28 patients with cerebral folate deficiency included 20 boys and 8 girls (median age at the time of the study, 7.1 years; range, 2.5 to 19.3), all of whom had received the diagnosis at the Division of Pediatric Neurology, University Hospital Aachen, in Aachen, Germany. The births and neonatal histories of these patients and the pregnancies of their mothers had been normal except in the case of one child (Patient 18), who had been born prematurely, at 28 weeks of gestation. All the parents were healthy and unrelated except for the parents of Patient 12, who were first cousins. The patients and their age-matched normal controls were not anemic, and their serum levels of vitamin B₁₂ and homocysteine were normal, as were their serum and erythrocyte levels of folate. Specimens of cerebrospinal fluid were obtained from the patients by lumbar puncture, and 5MTHF, the major form of folate in the cerebrospinal fluid, was measured by high-performance liquid chromatography with electrochemical detection and the results compared with values derived in our laboratory from 99 normal controls, as previously described (mean, 82 nmol per liter; range, 44 to 181). In addition to a reduced level of 5MTHF in the cerebrospinal fluid, the inclusion criteria required that each child have at least three of the major clinical findings characteristic of the cerebral folate deficiency syndrome. The age at the onset of the major clinical symptoms among the patients with cerebral folate deficiency is shown in Figure 1.

After the diagnosis of the cerebral folate deficiency syndrome had been established, treatment with folinic acid (0.5 to 1 mg per kilogram of body weight daily in two divided doses) was started. Patients were then examined at one, three, and six months and every six months thereafter. Six months after the start of treatment, lumbar puncture was repeated to determine the 5MTHF level in the cerebrospinal fluid, whereupon the dose of folinic acid was adjusted to maintain a normal level of folate in the cerebrospinal fluid.

The history and neurologic examination of the 28 control subjects (17 boys and 11 girls; median age, 7.6 years; range, 1.9 to 19.0) did not reveal any of the symptoms of the cerebral folate deficiency syndrome.

SERUM ASSAYS FOR AUTOANTIBODIES AGAINST FOLATE RECEPTORS
The procedure for identifying autoantibodies against membrane-bound folate receptors was modified by first incubating the serum with solubilized, purified folate receptors and then adding [³H]folic acid. Blocking autoantibodies, if present, prevent the binding of [³H]folic acid to folate receptors. All identifying information had been removed from the serum specimens, which were identified only after the assays had been completed and the results forwarded for analysis.

For this assay, 100 µl of serum was added to a button of dextran-coated charcoal to remove free folate. Aliquots of the serum (30 µl and 60 µl) were then incubated in a total volume of 500 µl of 0.01 M sodium phosphate buffer (pH 7.4) containing...
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patient No.</th>
<th>Rate of Improvement in Symptoms</th>
<th>% of subjects</th>
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<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
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<td>85</td>
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<tr>
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<tr>
<td>M</td>
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<td>+/−</td>
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<tr>
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<td>+/−</td>
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<td>Age at diagnosis (yr)</td>
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<tr>
<td>15</td>
<td>16</td>
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<td>Irritability, marked unrest, sleep disturbances</td>
<td>+/−</td>
<td>+/−</td>
<td>84</td>
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<td>Head-growth deceleration after 6 mo</td>
<td>+/−</td>
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<tr>
<td>Psychomotor retardation</td>
<td>+/−</td>
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<td>39</td>
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<td>Frank autistic features and late infantile autism†</td>
<td>−</td>
<td>−/−</td>
<td>40</td>
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<td>Cerebellar ataxia</td>
<td>−</td>
<td>−/−</td>
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<td>Wobbly gait, difficulty walking</td>
<td>−</td>
<td>−/−</td>
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<tr>
<td>Ataxia with frequent falls</td>
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<tr>
<td>Severe ataxia</td>
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<td></td>
</tr>
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<td>Pyramidal tract signs in legs</td>
<td>+/−</td>
<td>+/−</td>
<td>81</td>
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<td>Dyskinesias (e.g., choreoathetosis and ballismus)</td>
<td>−</td>
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<tr>
<td>Occasional seizures†</td>
<td>−</td>
<td>+/−</td>
<td>100</td>
</tr>
<tr>
<td>Epilepsy</td>
<td>+/−</td>
<td>+/−</td>
<td></td>
</tr>
<tr>
<td>Ocular strabismus†</td>
<td>−</td>
<td>+/−</td>
<td>33</td>
</tr>
<tr>
<td>Visual failure or optic atrophy†</td>
<td>−</td>
<td>+/−</td>
<td>0</td>
</tr>
<tr>
<td>Sensorineural hearing loss</td>
<td>−</td>
<td>+/−</td>
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</table>

* +/− denotes improvement with treatment, +/+ no improvement with treatment, − symptoms not present, and 0/+ decreased severity after treatment.
† This characteristic is a less common feature of cerebral folate deficiency.
0.5 percent Triton X-100 overnight at 4°C with 0.18 pmol of solubilized apo-folate receptor (which lacks bound folate) purified from human placental membranes. [3H]Folic acid was then added and the mixture incubated for 30 minutes at room temperature. Free [3H]folic acid was removed by adsorption to the dextran-coated charcoal, and receptor-bound radioactivity in the supernatant fraction was measured. [3H]Folic acid binds to the receptors in a 1:1 molar ratio, and the amount of radioactivity bound to the receptors is inversely related to the titer of the blocking autoantibodies and is expressed as picomoles of receptor blocked from binding [3H]folic acid, normalized to 1 ml of the serum assayed.

The amount of endogenous apo-folate–binding protein in each serum specimen was determined by the binding of [3H]folic acid, and this value was added to the 0.18 pmol of purified apo-folate receptor to determine the total amount of folate receptors blocked by the autoantibodies.

To establish that the autoantibodies were indeed immunoglobulins, 16 positive serum specimens were analyzed by adding sufficient protein A–trisacryl to bind four times the average level of IgG in serum. After a one-hour incubation, the protein A–trisacryl was pelleted; the supernatant fraction contained no immunoglobulins and showed no blocking activity. Dissociation of the immunoglobulins from the protein A–trisacryl was obtained by acidification, and this fraction, after neutralization, contained autoantibodies to folate receptors.

**BINDING AFFINITY STUDIES**

Purified apo-folate receptors from human placenta were prepared as previously described and incubated overnight at 4°C with serum containing autoantibodies from which free folate was removed by adsorption to the coated charcoal. [3H]Folic acid was then added, and the fraction bound to the folate receptors was subtracted from the total folate-binding capacity of the receptors to derive the quantity (in picomoles) of receptors blocked by the autoantibodies. Scatchard analysis of the ratio of the autoantibody-blocked receptor to the unblocked apo-folate receptor was used to compute the apparent association constant (K_a).

**STATISTICAL ANALYSES**

The percentage of children whose specimens tested positive for blocking autoantibodies against the folate receptors was compared with the expected distribution within the group with cerebral folate deficiency and the control group. Assuming an equal distribution of the number of positive autoantibody tests in the two groups, the chi-square value and its P value with one degree of freedom were calculated to test this hypothesis. The statistical analysis was also adjusted for sex. The two-tailed t-test for two independent samples was used to compare the age distribution and serum folate levels between the two age-matched groups. The aforementioned t-test was also used to compare pre-treatment and post-treatment levels of 5MTHF in the cerebrospinal fluid in the group with cerebral folate deficiency and our previously established reference data obtained from the 99 normal controls.

**RESULTS**

The patients with cerebral folate deficiency and their controls had similar distributions of age and sex (mean age difference, 3.3 months; range, 0 to 9.0). There was no significant difference with respect to their serum folate levels. Blocking autoantibodies against the folate receptors were identified in serum specimens from 25 of 28 children with cerebral folate deficiency and in 0 of 28 matched control subjects (P<0.001 by the chi-square test) (Table 2.
The mean titer of blocking autoantibodies in the serum of cerebral folate deficiency subjects was 0.87 pmol of folate receptor blocked per milliliter of serum. The mean apparent $K_a$ for the binding of these autoantibodies to the folate receptor was $5.54 \times 10^{10}$ liters per mole. Serum specimens from three children with cerebral folate deficiency (Patients 7, 9, and 21) did not contain these autoantibodies. Patient 9, who had four of the clinical criteria for the syndrome, had frank autistic behavior and recovered completely after receiving 400 µg of folic acid daily; he currently attends a regular school. He was the first child identified to have the syndrome and received a multivitamin containing folic acid, whereas all the other children were treated with folinic acid. Patients 7 and 21 also had remarkable improvements with folinic acid, although the changes were not as dramatic as those in Patient 9.

Among the 25 children with blocking autoantibodies, 4 (Patients 4, 16, 19, and 26) also fulfilled the conditions of late-infantile autism according to the Autism Diagnostic Observation Schedule criteria.

### Table 2. Characteristics of the Subjects and Laboratory Values.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients with Cerebral Folate Deficiency (N=28)</th>
<th>Age-Matched Controls (N=28)</th>
<th>Statistical Test Result</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male:female ratio</td>
<td>20:8</td>
<td>17:11</td>
<td>Chi-square=0.71</td>
<td>NS</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>Median</td>
<td>7.1</td>
<td>7.6</td>
<td>t value=0.083</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>2.5–19.3</td>
<td>1.9–19.0</td>
<td></td>
</tr>
<tr>
<td>Blocking folate receptor autoantibodies in serum (no. of subjects)</td>
<td>25</td>
<td>0</td>
<td>Chi-square=27.16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Titer (pmol of receptor blocked/ml)</td>
<td>Mean</td>
<td>0.87</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>0–1.55</td>
<td></td>
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</tr>
<tr>
<td>Affinity constant (liters/mol)†</td>
<td>Mean</td>
<td>$5.54 \times 10^{10}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>$0.88 \times 10^{10}$ to $15.40 \times 10^{10}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum folate (nmol/liter)</td>
<td>Mean</td>
<td>30.1</td>
<td>29.0</td>
<td>t value=0.22</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>10.2–54.4</td>
<td>8.3–45</td>
<td></td>
</tr>
<tr>
<td>5MTHF in cerebrospinal fluid (nmol/liter)</td>
<td>Before treatment</td>
<td>Mean</td>
<td>20.6‡</td>
<td>82‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Range</td>
<td>0–46.3</td>
<td>44–181</td>
</tr>
<tr>
<td>After folinic acid treatment</td>
<td>Mean</td>
<td>73.3</td>
<td>82‡</td>
<td>t value=1.24§</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>45.4–120.7</td>
<td>44–181</td>
<td></td>
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</tbody>
</table>

* NS denotes nonsignificant, and 5MTHF 5-methyltetrahydrofolate.
† The autoantibodies from Patients 13, 14, and 23 appeared to have two orders of binding sites on the folate receptor, whereas the remaining patients had a single order of autoantibody-binding sites on the receptor.
‡ The values were obtained from 99 healthy controls.
§ Pretreatment and post-treatment values were compared with values obtained for 99 healthy controls.
These four children with mental retardation associated with autism had very high titers of blocking autoantibodies (i.e., 1.27, 1.20, 0.65, and 1.27 pmol of folate receptor blocked per milliliter of serum). Treatment with folic acid or folinic acid improved the communication skills and neurologic abnormalities in the two younger autistic children, who received the diagnosis of cerebral folate deficiency at the ages of two and three years. The two older children with this diagnosis, who were treated beginning at the ages of 5 and 12 years, had a poorer outcome and remained autistic. Three brothers with cerebral folate deficiency (Patients 2, 17, and 20), who had a response to folinic acid treatment, had infantile-onset irritability, psychomotor retardation, and ataxia, and their serum contained blocking autoantibodies.

Epilepsy developed in six children, and two others (Patients 20 and 25) had occasional seizures; the former six had intractable epilepsy with absences, myoclonic atactic attacks, and grand mal seizures requiring anticonvulsant therapy. After the diagnosis of low cerebrospinal fluid folate levels had been established, folinic acid was added to their anticonvulsant treatment, after which the seizures were fully controlled. Patient 24 had intractable myoclonic atactic seizures, and her condition deteriorated despite treatment with both valproate and folinic acid. However, after the valproate was replaced with ethosuximide, she became seizure-free and recovered neurologically.

Pretreatment cerebrospinal fluid folate levels among the patients with cerebral folate deficiency were significantly lower (mean, 20.6 nmol per liter; range, 0 to 46.3) than the values obtained in the 99 normal subjects (P<0.001). After the administration of folinic acid, the cerebrospinal fluid folate levels in the patients normalized (mean, 73.3 nmol per liter; range, 45.4 to 120.7); the difference from the values in the normal controls was not significant, according to the t-test (Table 2).

Figure 2. Blocking Autoantibodies against the Folate Receptor in the Serum of Children with Cerebral Folate Deficiency and Age-Matched Control Subjects.

The mean (±SE) autoantibody titer was 0.87±0.08 pmol of folate receptor blocked per milliliter of serum. No autoantibodies were present in the serum of the age-matched control subjects.

The finding of blocking autoantibodies against folate receptors in serum from children with cerebral folate deficiency supports our hypothesis that this neurologic disorder can be a consequence of autoantibody-impaired folate transport into the cerebrospinal fluid. The high affinity of the autoantibodies (mean $K_a$ of $2.2\times10^{10}$ liters per mole) allows them to prevent folate from binding to the receptors on the epithelial cells of the choroid plexus. Since autoantibodies with a mean $K_a$ of $2.2\times10^{10}$ liters per mole were shown to block the binding and cellular uptake of $[^3H]$folic acid by KB cells,\textsuperscript{8} autoantibodies with a higher $K_a$, such as those in the serum from subjects with cerebral folate deficiency, would have a similar effect.

Autoantibodies against GPI-anchored folate receptors preferentially bind to epithelial cells on the plasma side of the choroid plexus. Folate receptors in the lungs and thyroid gland could be affected by these blocking autoantibodies. The folate receptors on the luminal side of the proximal renal tubules will not be affected because immunoglobulins do not pass into the renal tubules of normal kidneys.

There are three possible mechanisms by which folate enters the cerebrospinal fluid. First, treatment with pharmacologic doses of 5-formyltetrahydrofolate (folinic acid), most of which is enzymatically converted in vivo to the physiologically active 5MTHF,\textsuperscript{13,14} enters the cerebrospinal fluid by way of the reduced folate carrier on the choroid epithelial cells. A second pathway is displacement of blocking autoantibodies to the folate receptors by a high level of 5MTHF (approximately 2 µM or greater). A third mechanism could be diffusion, when the plasma level of 5MTHF is very high.

Because the first clinical manifestations of cerebral folate deficiency appear after the age of four to
six months and because the mothers we tested had no autoantibodies, the production of autoantibodies in these children probably occurred during the first four to six months of life. We speculate that the production of autoantibodies against the folate receptor could be induced by soluble folate-binding proteins in human or bovine milk or result from sensitization by unknown antigens with similar epitopes. Soluble-folate binding proteins in milk share amino acid sequence homology (91 percent similarity) with the membrane-bound folate receptors alpha and beta that are expressed on human choroid plexus epithelium. Moreover, the folate receptors on the choroid plexus cross-react with rabbit antibodies against the human-milk folate-binding protein. Autoantibodies against these epitopes could result in reduced folate transport into the cerebrospinal fluid.

Early detection and diagnosis of cerebral folate deficiency are important because folinic acid at a pharmacologic dose and the 5MTHF derivative can bypass autoantibody-blocked folate receptors and enter the cerebrospinal fluid by way of the reduced folate carrier. This route restores the folate level within the central nervous system and can ameliorate the neuropsychiatric disorder.

Supported in part by a research grant from the Medical Faculty Aachen, by a grant from the Swiss National Science Foundation (3100-066953.01, to Dr. Blau), and by the Dr. Emil-Alexander Hübner Foundation (both to Dr. Opladen).

We are indebted to the parents of the affected children; to the doctors and members of the nursing staff of the Department of Pediatrics, University Hospital Aachen, for their support during this study; to the members of the nursing staff of the Division of Pediatric Neurology, for their care of children with neurometabolic and genetic diseases; and to Lucja Kierat, without whose help the cerebrospinal fluid analyses for this study would not have been possible.

REFERENCES