Multiple sclerosis (MS) most frequently manifests as a relapsing-remitting (RR) disorder which evolves into a secondary progressive (SP) phase in up to 50% of patients. The primary progressive (PP) form of the disease, which is observed in about 10% of the patients, is characterized by a progressive course from onset with no history of relapse or remission. PP MS patients exhibit smaller and fewer magnetic resonance imaging (MRI) lesions than do SP and RR patients. An estimated 30% of PP patients do not show new or enlarging central nervous system lesions on serial T2-weighted scan studies but still display a progressive decline in Expanded Disability Status Scale (EDSS) score, suggesting a lack of correlation between clinical scores and MRI scans.

The purpose of the current study was to compare lymphocyte migration and IFN-γ production by peripheral blood T lymphocytes obtained from PP MS subgroups based on the presence of high- or low-lesion volume on their T2-weighted MRI scans. We found a positive correlation between these in vitro measures and T2-weighted lesion volume in PP MS. This correlation between immune function and MRI suggests that the heterogeneity of immune mechanisms is linked with disease pathogenesis.

Methods

Patients and MRI

A total of 11 PP MS patients were included in this study (Table). We used criteria reported by Lublin and co-workers to define PP MS. Patients were preselected from a larger PP MS cohort being considered for clinical trials on the basis of their lesion volumes on previously performed MRI scans. Three patients with progressive spastic paraparesis (PSP) and 10 healthy donors were used as controls. Proton MRI examinations of the brain were obtained using a Philips Gyroscan ACSII operating at 1.5 T (Philips Medical Systems, Best, The Netherlands). A transverse dual-echo, turbo spin-echo sequence (TR/TE1/TE2 = 2075/32/90 msec, 256 × 256 matrix, 1 signal on average, 250-mm field of view) yielding proton density (PD)–weighted and T2-weighted images with 50 contiguous 3-mm slices was acquired parallel to the line connecting the anterior
and posterior commissures, followed by a matching T1-weighted sequence (TR/TE = 35/10 msec). Lesion volumes were manually segmented using locally developed software (Display, developed by David MacDonald; Brain Imaging Center, Montreal Neurological Institute, Montreal, Quebec, Canada), which provides simultaneous access to PD-, T2-, and T1-weighted image sets. Lesion boundaries were primarily determined on the PD-weighted images. MRI and blood collection were done on the same day, and patients were subdivided a posteriori into two categories according to the volumetric quantification of lesions done on the T2-weighted MRI scans: either high (>10 mm³) or low lesion load volume (<3 mm³).

**Boyden Chamber Assay**

These assays were performed in Boyden chambers (3-μm pore size membranes) pre-coated with fibronectin (Collaborative Biomedical Products, Bedford, MA) as previously described. A total of 7 × 10⁵ cells (≥95% CD3⁺ T cells) in 700 μl of RPMI plus 2.5% fetal calf serum (Medicorp, Montreal, Quebec, Canada) was added to the upper chamber; the lower chamber was filled with 1 ml of RPMI plus 10% fetal calf serum (Medicorp). After 6 hours of incubation at 37°C, cells from the lower chamber were collected and counted using a hemocytometer.

**Intracellular Cytokine Staining**

This assay was performed using a 4-hour incubation with brefeldin A (10 μg/ml), ionomycin (1 μg/ml), and phorbol myristate acetate (25 ng/ml) (all from Sigma, Mississauga, Ontario, Canada) as previously described. Cells were stained for 30 minutes with anti-CD3 monoclonal antibody conjugated to phycoerithrine (Becton-Dickinson, Mississauga, Ontario, Canada), fixed in 4% paraformaldehyde, and stained for an additional 30 minutes with anti–IFN-γ monoclonal antibody conjugated to FITC (Becton-Dickinson). Immunofluorescence was detected using a Becton-Dickinson flow cytometer and analyzed using a WinMDI program (Scripps, La Jolla, CA).

**Statistical Analysis**

One-way ANOVA and the Dunett posttest were used to compare the different groups; n represents the number of patients in each group. ANOVA, linear regression, and correlation coefficients were obtained with the Prism-Graph Pad program (version 2.0). Each migration data result represents the mean of duplicate experiments.

**Results**

**Patients**

As shown in the Table, the high and low lesion volume groups did not differ with regard to mean disease duration or mean disability scores. No difference in rates of disease progression (EDSS/disease duration) was observed.

**Migration of PB T Lymphocytes Correlates with Lesion Load Volume on T2-Weighted Scans**

As shown in Figure 1, the migration rates of PB T lymphocytes obtained from PP MS patients with a high lesion load on their MRI scans were significantly higher than the rates of migration of T cells obtained from healthy donors and PSP patients (p < 0.005). The migration rates of lymphocytes derived from PP MS patients with low lesion volumes were significantly lower than those of PP MS patients with a high lesion burden and not different from T-cell migration rates of healthy controls and PSP patients. Linear regression between migration and T2-weighted lesion volume in our PP MS population showed a correlation coefficient of 0.9505 (r²) (p < 0.001).

**IFN-γ–Secreting Cells in PB T Lymphocytes from PP MS and Healthy Controls**

Figure 2 compares the PB T-cell donor subgroups with regard to IFN-γ–secreting CD3⁺ cells (the overall T-lymphocyte population). Values of IFN-γ–secreting CD3⁺ cells were significantly increased in PP MS patients, who showed a high lesion load burden compared with healthy controls, and PSP patients (p < 0.02). PP MS patients with low lesion volumes had values of IFN-γ+/CD3⁺ T cells comparable to those of controls and PSP patients. Linear regression between the percentage of IFN-γ–producing T cells and T2-weighted lesion volume in the PP MS population

### Table. Mean Age, Disability Scores (EDSS), T2-Weighted Lesion Volume, and Disease Duration of Patient Groups and Controls

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Age, yr (range)</th>
<th>Female/Male Ratio</th>
<th>EDSS (range)</th>
<th>Disease Duration, yr (range)</th>
<th>Progression Rates</th>
<th>T2-Weighted Lesion Volume, cm³ (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>10</td>
<td>34.2 (19–54)</td>
<td>6/4</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>PSP</td>
<td>3</td>
<td>51.3 (47–56)</td>
<td>0/3</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>PP MS with low lesion</td>
<td>6</td>
<td>46.2 (37–58)</td>
<td>4/2</td>
<td>5.6 (3.0–8.5)</td>
<td>12.5 (5.0–15.5)</td>
<td>0.44</td>
<td>1.45 (0.3–2.4)</td>
</tr>
<tr>
<td>volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP MS with high lesion</td>
<td>5</td>
<td>52.1 (46–63)</td>
<td>3/2</td>
<td>6 (3.0–6.5)</td>
<td>15.5 (7.5–19.0)</td>
<td>0.39</td>
<td>15.3 (10.1–25.3)</td>
</tr>
</tbody>
</table>

Patient groups are composed of patients with primary progressive (PP) multiple sclerosis (MS) with either a high or low T2-weighted lesion volume or patients with progressive spastic paraparesis (PSP). EDSS = Expanded Disability Status Scale; NA = not applicable.
showed a correlation coefficient of 0.6093 (r^2) (p < 0.005).

**Discussion**

In this study, we used Boyden chambers to evaluate the migratory behavior of T cells and intracellular cytokine staining to assess the number of PB T cells expressing IFN-γ in PP MS patients. We preselected patients based on lesion volume on T2-weighted MRI. The high and low PP patient subgroups did not differ with regard to mean rate of disease progression (EDSS/disease duration). We found that the patients with a high
T2-weighted lesion load had a significantly higher migration rate compared with patients with a low lesion load and controls, suggesting that in vitro lymphocyte migration could serve as marker for the rate of accumulation of the MRI-defined burden of disease. Furthermore, we could show a significant correlation between the rate of migration and lesion volume in the overall PP MS population. Although Leppert and colleagues previously showed that RR and PP MS patients have increased MMP-9 levels in their cerebrospinal fluid, they did not provide a correlation between MRI profile and MMP levels.

Our intracellular cytokine data indicate that a significantly higher number of T cells expressing IFN-γ can be found in the blood of PP MS patients carrying a high lesion burden compared with PP MS patients with a low lesion volume, patients with PSP, and healthy controls. This increase in the number of IFN-γ–secreting cells was also correlated with the level of MRI-defined T2-weighted lesion load. T cells derived from SP MS patients were previously shown to have a higher number of IFN-γ–positive T cells compared with controls. The high number of IFN-γ–positive T cells could reflect the effect of the monocyte-derived interleukin-12 cytokine, which has been shown to be increased in MS and is known to polarize T cells toward a Th1 phenotype.

IFN-β and GA are currently used for the treatment of RR MS, reducing both relapses and disease progression. IFN-β has also been shown to be effective in delaying disease progression in SP MS. The effects of immunomodulatory medication on the PP MS disease course are now under investigation. We have previously shown that RR MS and SP MS patients are 2 homogeneous groups in terms of migration and number of IFN-γ–secreting cells. This report highlights the fact that PP MS patients are heterogeneous in terms of cellular immune function and that their immunological markers correlate with the extent and rate of development of lesion burden on MRI scans. The cellular immune functions did not correlate with clinical measures of disease duration or severity. Ongoing clinical trials will establish whether immunological and MRI parameters correlate with therapeutic response in PP MS patients.

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References
No Acute Antimigraine Efficacy of CP-122,288, a Highly Potent Inhibitor of Neurogenic Inflammation: Results of Two Randomized, Double-Blind, Placebo-Controlled Clinical Trials

K. I. Roon, MD,* J. Olesen, MD, PhD,† H. C. Diener, MD, PhD,‡ P. Ellis, PhD,§ J. Hettiarachchi, MD, FRCP,§ P. H. Poole, CStat,§ I. Christianssen, MD,† D. Kleinermans, MD, PhD,‖ J. G. Kok, MD,§ and M. D. Ferrari, MD, PhD*  

CP-122,288 is a highly potent inhibitor of neurogenic plasma extravasation in animal models at doses without vasoconstrictor effect. We evaluated the acute antimigraine efficacy of intravenous and oral CP-122,288 in two double-blind studies. In a crossover design, patients randomly received 31.25 µg of CP-122,288 intravenously, placebo, or both. In the oral study, patients received placebo or one of four doses of CP-122,288 between 3.125 and 312.5 µg, using a novel “up and down” design for randomization. Both studies were stopped prematurely when target efficacy could not be achieved. Responder rates were 29% for CP-122,288 versus 30% for placebo (difference, 1%; 95% CI, −24–22%; intravenous study) and an overall rate of 25% for CP-122,288 versus 0% for placebo (difference, 25%; 95% CI, 10–40%; oral study). CP-122,288 was not clinically effective at doses and plasma concentrations in excess of those required to inhibit neurogenic plasma extravasation in animals. Neurogenic plasma extravasation is unlikely to play a crucial role in the pathophysiology of migraine headache.


From the *Department of Neurology, Leiden University Medical Centre, Leiden, and #Department of Neurology, Gemini Hospital, Den Helder, The Netherlands; †Department of Neurology, Glostrup Hospital, Copenhagen, Denmark; §Neurologische Universitätsklinik, Essen, Germany; ‡Central Research, Pfizer, Sandwich, Kent, UK; and ‖Pfizer Clinical Research Unit, Erasmus Hospital, Brussels, Belgium.

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Address correspondence to Dr Ferrari, Department of Neurology, K5Q Leiden University Medical Centre, PO Box 9600, 2300 RC Leiden, The Netherlands.

Coronary vasoconstriction is a major drawback of all current specific antimigraine compounds. Selective neuronal inhibition within the trigeminalvascular system without associated vasoconstrictor activity would be a major improvement for the safety of antimigraine drugs. Inhibition of neurogenic plasma extravasation (NPE) in animal models is believed to predict antimigraine efficacy mediated via selective neuronal action.

CP-122,288 blocks NPE within rat guinea pig dura mater in a dose-dependent manner at doses several thousand times smaller than those required to cause vasoconstriction. The threshold to inhibit NPE in rat dura mater is 3 ng/kg administered intravenously, and the thresholds to induce vasoconstriction of the dorsal hand vein in man are 125 µg (intravenously) and 1 mg (orally).

To assess the contribution of NPE to migraine pathophysiology, we evaluated the efficacy of CP-122,288 in the acute treatment of migraine in two randomized, double-blind, placebo-controlled, “proof of concept” studies. In one trial with a crossover design, we compared the efficacy of intravenous placebo and 31.25 µg of CP-122,288. In the other trial, we employed a novel “up and down” randomization system to cover a wide range of oral doses. In both trials, CP-122,288 doses were above the threshold for inhibition of NPE in animals and below the threshold for vasoconstriction in man.

Methods
Migraine patients with or without aura (attack frequency, 1–12 every 2 months) between 18 and 65 years of age were recruited from outpatient departments of six centers in Denmark, Belgium, and the United Kingdom (intravenous study) and eight centers in the Netherlands and Germany (oral study). Patients were excluded if they had nonmigrainous headache for more than 10 days/mo, were known nonresponders to antimigraine medication, had a history of serious disorders, were abusing alcohol and/or drugs (including analgesics, sumatriptan, or ergots), or were on regular medication. Prophylactic treatment had to be stopped more than 2 weeks prior to study treatment. Patients were screened less than 16 (intravenous study) or 8 (oral study) weeks prior starting the study medication. On the study day, the study medication was administered less than 12 (intravenous study) or 6 (oral study) hours after the onset of moderate or severe headache and if no ergots, sumatriptan, or analgesics had been taken within the previous 48, 24, or 12 hours, respectively.

In a double-blind, randomized, placebo-controlled, two-period, crossover, inpatient study, patients received 31.25 µg of CP-122,288 intravenously or placebo during two migraine attacks separated by at least 1 week. In a double-blind, placebo-controlled, parallel-group, single-attack, inpatient study, patients were allocated to placebo or oral 0.625, 3.125, 12.5, 62.5, or 312.5 µg of CP-122,288 according to a novel up and down system for randomization. The first patient in the trial received either 12.5 µg of CP-122,288 (ie, the middle dose) or placebo. Two hours after treatment,
a central computer was called to report the patient's response: nonresponse was to be followed by a higher dose for the next patient in the trial, and a good response was to be followed by a lower dose for the next patient. This procedure was repeated for all subsequent patients. A placebo was inserted at random (1 in 4 patients). Responses to placebo or in patients who vomited within 30 minutes were not included in the allocation process or in the assessments. Plasma samples of CP-122,288 were taken at 2 hours postdose.

Headache severity, associated symptoms, and tolerability were assessed on a four-point scale (severe, moderate, mild, none) every 30 minutes postdose. After 2 hours, all patients could use escape medication, the screening investigation was repeated, and patients left the hospital, recording their migraine symptoms on diary cards for up to 24 hours after dosing. They were interviewed by telephone within 24 to 48 hours and re-examined within 5 to 9 days.

Primary efficacy measures were (1) response at 2 hours posttreatment (ie, improvement from moderate or severe to mild or no pain) and (2) pain-free at 2 hours. Secondary end points were improvement of associated symptoms, use of rescue medication, and recurrence of headache within 24 hours.

Assuming responder rates of 30% (placebo) and 65% (CP-122,288) and at $\alpha = 0.05$ and $\beta = 0.2$, sample size calculations indicated that 32 patients were required in each treatment group for the intravenous study and 12 patients were required in each of the oral study groups.

Both studies were conducted in accordance with the Declaration of Helsinki and the Principles of Good Clinical Practice. Approval was obtained from the local ethics committees, and informed consent was obtained from each patient before enrollment.

### Results

A blind interim analysis triggered by low overall response rates confirmed the absence of a potential clinically relevant efficacy for CP-122,288 (≥50% of absolute response and 30% difference from placebo). Both studies were therefore terminated for ethical reasons, and a formal intention-to-treat analysis was performed.

Demographic and clinical characteristics of the study populations and attacks were comparable between groups and trials (Table 1). CP-122,288 was well tolerated, and adverse events were mild or moderate, short-lived, and comparable between groups.

In the intravenous study, 22 patients completed the crossover, 6 received CP-122,288 only, and 8 received placebo only (ie, 28 attacks treated with CP-122,288 and 30 with placebo). In the oral study, 12 patients received placebo and 32 different doses of CP-122,288, mostly the highest dose (Fig). There were no significant differences between the primary (Table 2; pain-free rates with active medication vs placebo: 0/28 and 1/30 [intravenous study] and 2/17 and 0/12 [oral study]) and secondary end points. CP-122,288 plasma concentrations after a 312.5-mg dose (orally) (mean, 0.46 ng/ml; range, 0.01–1.52 ng/ml) were greater than those required to inhibit NPE in animals.3,4

### Discussion

We tested CP-122,288, the most potent inhibitor of NPE in animal models currently available, in two differently designed and independently conducted clinical trials. Absence of clinical antimigraine efficacy was un-

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**Table 1. Demographic and Clinical Characteristics of the Study Populations and Study Attacks of the Intravenous Study (n = 36) and Oral Study (n = 44)**

<table>
<thead>
<tr>
<th></th>
<th>Intravenous Study</th>
<th>Oral Study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Active-Placebo (n = 18)</td>
<td>Placebo-Active (n = 18)</td>
</tr>
<tr>
<td>Sex ratio F:M</td>
<td>15:3</td>
<td>14:4</td>
</tr>
<tr>
<td>Age (yr)*</td>
<td>40 ± 11</td>
<td>40 ± 10</td>
</tr>
<tr>
<td>Migraine history</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age of migraine onset (yr)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migraine with aura</td>
<td>17 ± 8</td>
<td>19 ± 11</td>
</tr>
<tr>
<td>Without aura</td>
<td>11 (61%)</td>
<td>11 (61%)</td>
</tr>
<tr>
<td>Both</td>
<td>6 (33%)</td>
<td>4 (22%)</td>
</tr>
<tr>
<td></td>
<td>Active (n = 28)</td>
<td>Placebo (n = 30)</td>
</tr>
<tr>
<td>Pretreatment duration (hr:min)*</td>
<td>5:04 ± 1:52</td>
<td>5:38 ± 2:26</td>
</tr>
<tr>
<td>Headache severity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>13 (46%)</td>
<td>14 (47%)</td>
</tr>
<tr>
<td>Severe</td>
<td>15 (54%)</td>
<td>16 (53%)</td>
</tr>
</tbody>
</table>

*aExpressed as mean ± SD.*
equivocally shown at concentrations blocking NPE in animal models but lacking vasoconstrictor activity in man. In addition, we describe a novel clinical trial design for proof of concept studies. Compared with traditional study designs, the up-and-down design provides a much quicker answer to the question of whether an experimental drug is effective, exposing fewer patients and therefore being more cost-effective and ethical.

Lack of efficacy as the result of a type II error was highly unlikely for the following reasons: (1) the CIs for the response rates and the lack of differences from placebo make a clinically relevant effect of CP-122,288 unlikely; (2) low oral resorption seems unlikely, because the plasma concentrations after the highest dose of CP-122,288 were comparable to those in healthy volunteers and above the levels producing inhibition of NPE in animal studies; and (3) the clinical characteristics were similar among the different study groups and to those seen in other clinical trials with antimigraine compounds.

The findings with CP-122,288 corroborate those found in migraine trials evaluating the efficacy of the endothelin receptor antagonist bosentan; the substance-P (neurokinin-1) receptor antagonists GR205171, L-758,298, lanepitant, and RPR-100893; and the neurosteroid modulator ganaxolone. All these drugs inhibit NPE, are devoid of vasoconstrictor activity, and failed to show antimigraine efficacy. Thus, inhibition of NPE in animal models does not reliably predict clinical efficacy of experimental antimigraine drugs, and NPE is unlikely to be a crucial role in the pathophysiology of the fully established migraine attack.

In conclusion, CP-122,288 failed to demonstrate clinical efficacy at doses in excess of those required to inhibit NPE in animals. Inhibition of NPE in animal models does not reliably predict clinical efficacy of experimental antimigraine drugs, and NPE is unlikely to be a crucial role in the pathophysiology of the fully established migraine attack.
play a crucial role in the pathophysiology of migraine headache.

Appendix

The CP-122,288 Migraine Study Group consisted of the following (in order of number of patients contributed):

**Intravenous study:** J. Olesen and I. Christianssen, Department of Neurology, Glostrup Hospital, Copenhagen, Denmark; D. Kleinermans, Hospital Erasme, Brussels, Belgium; J. McEwen, Drug Development Scotland, Ninewell Hospital and Medical School, Dundee, Scotland; T. Staelin-Jensen, Department of Neurology, Arhus Kommunehospital, Denmark; T. J. Steiner, The Princess Margaret Migraine Clinic, London, UK; M. D. Eve, Pfizer Clinical Research Unit, Kent and Canterbury Hospital, Canterbury, UK.

**Oral study:** M. D. Ferrari and K. I. Roon, Department of Neurology, Leiden University Medical Centre, Leiden, The Netherlands; J. G. Kok, Department of Neurology, Gemini Hospital, Den Helder, The Netherlands; A. Beckman-Reinholdt, Migräneklinik Königstein, Germany; H. D. Beikufner, Eckerndorfe, Germany; J. Haan, Department of Neurology, Rijnland Hospital, Leiderdorp, The Netherlands; H. C. Diener and A. Gendolla, Department of Neurology, University Hospital Essen, Germany; S. Strache, Berlin, Germany; M. Föh, Fulda, Germany; P. Ellis, J. Hettiarachchi, and P. Poole, Central Research Pfizer, Sandwich, UK.

This study was supported financially by Pfizer Limited, Sandwich, Kent, UK.

This paper was presented in preliminary form at the Eighth Congress of the International Headache Society in Amsterdam, June 1997 (Roon KI, Dienek HC, Ellis P, et al. CP-122,288 blocks neurogenic inflammation, but is not effective in aborting migraine attacks: results of two controlled clinical trials. Cephalalgia 1997;17:245 [Abstract]).

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Significant Association between the tau Gene A0/A0 Genotype and Parkinson’s Disease

Pau Pastor, MD,* Mario Ezquerra,† Esteban Muñoz, MD,* María José Martí, MD, PhD,* Rafael Blesa, MD, PhD,‡ Eduard Tolosa, MD, PhD,* and Rafael Oliva, MD, PhD†

A significant association between the tau gene A0/A0 genotype and progressive supranuclear palsy has been reported recently. To determine if the presence of a tau polymorphism could constitute a risk factor for the development of sporadic and familial Parkinson’s disease, a dinucleotide repeat marker at intron 11 was genotyped in 152 patients with PD, 52 patients with Alzheimer’s disease, and 150 healthy controls. We detected a significant difference in A0 allelic frequency in the Parkinson’s disease group (79.27%) compared with the control group (71%) and the Alzheimer’s disease group (73.07%). Individuals homozygous for the A0 allele were also detected significantly more frequently in the Parkinson’s disease group (63.8%) compared with the control group (52.66%) and the Alzheimer’s disease group (48.07%). These results suggest a possible involvement of the tau gene in the pathogenesis of some cases of Parkinson’s disease.

The etiology of Parkinson’s disease (PD) and other parkinsonian disorders is unknown, but increasing evidence suggests that genetic and environmental factors may be involved in the pathophysiology of PD. Progressive supranuclear palsy (PSP) is a parkinsonian disorder for which it has been demonstrated in independent series that polymorphisms of the tau gene behave as risk factors.1–3 Tau is a phosphoprotein that binds to microtubules and promotes their polymerization and stability. Neurofibrillary tangles (NFTs), which are mainly composed of a hyperphosphorylated form of tau, are found in many neurodegenerative diseases such as Alzheimer’s disease (AD), PSP, corticobasal degeneration, and frontotemporal dementia among others.4 NFTs are not the main pathological hallmark of PD, while the presence of Lewy bodies (LBs) is one of the mandatory histological characteristics. However, there are several biochemical and pathological findings that support a potential involvement of the tau protein in PD.5–7 We initiated this study to determine whether the tau gene dinucleotide polymorphism confers susceptibility to the development of PD in our population.

Subjects and Methods

A total of 152 patients (68 women and 84 men) with PD, 52 with AD (27 women and 25 men), and 150 healthy controls (80 women and 70 men) were included in this study. The PD patients (mean age of onset, 56.65 ± 11 years) were recruited from the Hospital Clinic Universitari (Barcelona) between 1996 and 1998. All met the UK Parkinson Society Brain Bank criteria for definite clinical PD.8 In 15 patients, the disease had an early onset (<40 years), and in 14 patients, the disease had a late onset (>70 years). The diagnosis of familial PD was made using the criteria proposed by Marder and co-workers.9 Forty-nine patients had a positive familial history for PD; in 13 cases, the familial history was uncertain, although 90 cases were sporadic. PD cases were classified as tremoric (n = 27), rigid-akinetic (n = 38), or mixed (n = 87).10 Clinical assessment was made using the Unified Parkinson’s Disease Rating Scale (UPDRS), the Hoehn and Yahr stage, and the Schwab and England Scale.11 Cognition was assessed by a brief mental status examination using the UPDRS scale (part I: mentation, behavior, and mood; 1. intellectual impairment).11 The AD patients (mean age of onset: 65.85 ± 10 years) were diagnosed with probable AD using NINCDS-ADRDA criteria.12 The controls (average age of controls: 54.50 ± 20 years) included healthy spouses of the patients with neurologological disorders (n = 83), nonaffected siblings of patients with myoclonic epilepsy (n = 18), and healthy individuals from the blood bank of the same hospital (n = 49). None of the 150 healthy controls had a familial history of parkinsonism. The present study was approved by the Ethics Committee of the Hospital Clinic Universitari (Barcelona).

Blood samples were drawn after obtaining informed consent, the DNA was isolated, and the intronic polymorphism was genotyped as described.1 Subsequently, we compared the genotype and allelic frequencies of the different groups and searched for correlations between the tau intronic polymorphism and familial or sporadic status, age of onset, mental status, and clinical subtype of PD. In addition, we tested 128 PD patients for the tau gene polymorphisms corresponding to exons 1 and 13. Statistical assessments were made using the SSPS 6.1 statistical package (SSPS, Chicago IL) and the χ² analysis for 2 × 2 tables.

From the *Parkinson’s Disease and Movement Disorders Unit, Neurology Service, Hospital Clinic Universitari, Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), †Genetics Service, Hospital Clinic Universitari and Human Genome Research Group, Faculty of Medicine, University of Barcelona, IDIBAPS, and ‡Neurology Service, Hospital Clinic Universitari, IDIBAPS, Barcelona, Spain.

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Address correspondence to Dr Oliva, Genetics Service, Hospital Clinic Universitari, Villarroel 170, 08036 Barcelona, Spain.
Results
No significant differences were found in the A0/A0 genotype frequency between the AD patients and the control group (Table 1). In contrast, a significantly higher frequency of A0/A0 genotype–positive individuals (63.8%) was found in the PD group compared with the control group ($p = 0.049$) and the AD group ($p = 0.045$).

The differences between PD patients and controls are also significant if the allelic frequency is considered instead of genotypes (see Table 1). Thus, we also found an increased frequency of the A0 allele in the PD patients (79.27%) compared with controls ($p = 0.018$; see Table 1). No significant differences were detected in the intronic allelic frequencies between AD patients and controls (see Table 1). The genotyping results corresponding to the polymorphisms present in exons 1 and 13 indicate a correlation of 98.7% between the H1 haplotype and the A0 allele corresponding to the intronic polymorphism. In addition, because the H1 haplotype corresponds to the A0 plus A1 alleles, we have considered the pooled data for both alleles as the basis for comparison (see Table 1). As shown, the differences remain significant in PD patients compared with controls (see Table 1).

A higher representation of the A0/A0 genotype is present in the familial group (69.4%) compared with the group with a sporadic presentation (60%) (Table 2). The frequency of the A0/A0 genotype and the A0 allele in the familial PD group is also significantly higher compared with the control group ($p = 0.04$ and $p = 0.022$, respectively; see Table 2). When the patients with PD were classified according to the age of onset of their symptoms, we found a significant increase in A0/A0 frequency in both the early-onset and late-onset groups ($p = 0.020$; see Table 2). This over-representation was not observed in the control group when the subjects were stratified according to age (see Table 2).

When the mental status of the PD patients was examined, we found that 23 patients suffered intellectual impairment, which was mild in 14 patients (scores 1 and 2 from UPDRS) and severe in 9 patients (scores 3 and 4 from UPDRS), although 129 patients had a normal mental status. Twenty-one patients (91.3%) with intellectual impairment had a disease duration longer than 10 years. Subsequently, the distribution of genotypes in individuals with 10 or more years of evolution of PD ($n = 93$) was evaluated, but we did not find any significant differences in the genotype frequencies based on the absence ($n = 72$) or presence ($n = 21$) of mental impairment (see Table 2). When the PD patients were classified according to other types of clinical presentation, no statistical differences were detected in either the allelic or genotype frequencies (see Table 2).

Discussion
We report a significantly increased frequency of the tau A0/A0 genotype and of A0 allelic frequency in patients with PD compared with either controls or patients

| Table 1. Allelic and Genotype Frequencies of tau Intronic Polymorphism in the Control Group, Parkinson’s Disease Group and Alzheimer’s Disease Group |
|---------------------------------|----------------|----------------|
| Genotypes                      | PD             | AD             | Controls        |
| A0A0                            | n = 152        | p = 0.049$^a$  | n = 150         |
| A1A0                            | 4 (2.6%)       | 3 (5.7%)       | 4 (2.6%)        |
| A2A0                            | 3 (2.0%)       | 0              | 1 (0.6%)        |
| A2A2                            | 0              | 0              | 3 (2.0%)        |
| A3A0                            | 38 (25.0%)     | 23 (44.2%)     | 50 (33.3%)      |
| A3A1                            | 1 (0.7%)       | 0              | 2 (1.3%)        |
| A3A2                            | 0              | 0              | 1 (0.6%)        |
| A3A3                            | 7 (4.6%)       | 1 (2.0%)       | 10 (6.6%)       |
| A4A0                            | 2 (1.3%)       | 0              | 0              |
| A0A0 + A0A1                     | 101 (66.44%)   | p = 0.047$^b$  | 83 (55.33%)     |
| Alleles                         | n = 304        | n = 104        | n = 300         |
| A0                              | 241 (79.2%)    | 76 (73.1%)     | 213 (71.0%)     |
| A1                              | 5 (1.6%)       | 3 (2.9%)       | 6 (2.0%)        |
| A2                              | 3 (1.0%)       | 0              | 8 (2.6%)        |
| A3                              | 53 (17.4%)     | 25 (24.0%)     | 73 (24.3%)      |
| A4                              | 2 (0.7%)       | 0              | 0              |
| A0 + A1                         | 246 (80.9%)    | p = 0.02$^d$   | 79 (75.96%)     |

$^{a}$Genotype A0A0 versus the rest of the genotypes.
$^{b}$Genotypes A0A0 plus A0A1 versus the rest of the genotypes.
$^{c}$Allele A0 versus A1, A2, A3, and A4 alleles.
$^{d}$Alleles A0 plus A1 versus A2, A3, and A4 alleles.

PD = Parkinson’s disease; AD = Alzheimer’s disease.
with AD. We have also considered whether misdiagnosis of some PD cases may have enriched the PD group with a substantial number of atypical parkinsonian disorders such as PSP. Our PD patients were diagnosed according to validated clinical criteria, however, and the patients with atypical parkinsonism were carefully excluded. Clinical misdiagnosis of PD is not infrequent (18%), but when applying the strict criteria of asymmetrical onset and no evidence of another disorder, misdiagnosis may be lower than 7%. Our patients fulfilled these additional criteria; thus, misdiagnosing of the sample examined is unlikely. In addition, when we corrected for up to an estimated 7% clinical contamination of PD with a highly unlikely 7% incidence of PSP, the A0 allele frequency in the PD group remained significant ($p = 0.018$ increase to $p = 0.049$).

The increased A0/A0 frequency in our series is consistent with a significant overrepresentation of the A0/A0 genotype as reported at a recent meeting in a limited series of 46 families with multicase PD (73.9% A0/A0 in PD vs 50% A0/A0 in controls). In addition, a higher frequency of the A0/A0 genotype in PD patients (58%) compared with controls (53%) has been reported recently in an independent population (n = 50), although the difference did not reach statistical significance. If the data reported by us are pooled with the data reported by Morris and colleagues, the difference detected between PD patients and controls is of statistical significance ($p = 0.013$). Interestingly, the frequency of the A0/A0 genotype is higher in our familial PD group (69.4%) compared with the sporadic PD group (60%). Overall, these independent studies suggest that the A0/A0 genotype could be a risk factor for PD, regardless of the genetic background of the respective populations.

The finding of this association was unexpected because of the lack of tau deposits in most PD patients. This prompted us to search for potential published evidence that the tau protein could be involved in the pathological process of PD. We found that a high incidence (42–85%) of cortical NFTs and senile plaques in PD, which increases (75–94%) when dementia is present, has been reported. It has been also reported that the LBs present in diffuse LB disease stain with an antibody to the microtubule-associated protein tau, although the LBs present in PD do not. Tau immunoreactive deposits have been detected in demented PD patients. We did not find a significant overrepresentation of the A0 allele in the PD patients with mental impairment, although it would be interesting to examine this point using a larger sample. Some cases with a clinical picture of PD have shown NFTs, neuronal loss, and gliosis (but no LBs) confined to the substantia nigra and locus ceruleus.

We also considered whether tau deposits are needed at all to define a specific disease. Another tauopathy is

### Table 2. tau Genotype and Allelic Frequencies in Parkinson’s Disease and in Controls Divided into Different Subgroups

<table>
<thead>
<tr>
<th>Genotypes n (%)</th>
<th>Alleles n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A0/A0</td>
<td>Others</td>
</tr>
<tr>
<td>PD patients (n = 152)</td>
<td>97 (63.8)</td>
</tr>
<tr>
<td>Age at onset*</td>
<td></td>
</tr>
<tr>
<td>≤40 years (n = 15)</td>
<td>13 (86.7)</td>
</tr>
<tr>
<td>41–70 years (n = 123)</td>
<td>72 (58.5)</td>
</tr>
<tr>
<td>&gt;70 years (n = 14)</td>
<td>12 (85.7)</td>
</tr>
<tr>
<td>Controls (n = 150)</td>
<td>79 (52.66)</td>
</tr>
<tr>
<td>≤40 years (n = 40)</td>
<td>21 (52.5)</td>
</tr>
<tr>
<td>41–70 years (n = 80)</td>
<td>46 (57.5)</td>
</tr>
<tr>
<td>&gt;70 years (n = 30)</td>
<td>12 (40.0)</td>
</tr>
</tbody>
</table>

Familial (n = 49)b | 34 (69.4) | 15 (30.6) | 81 (82.65) | 17 (17.34) |
Sporadic (n = 90)   | 54 (60.0) | 36 (40.0) | 139 (77.2) | 41 (22.7) |
Unclear (n = 13)    | 9 (69.2)  | 4 (30.8)  | 21 (80.7)  | 5 (19.2)  |

Tremoric (n = 27)   | 20 (74.1) | 7 (25.9)  | 47 (87.0)  | 7 (12.9)  |
Rigid-akineti (n = 38) | 25 (65.8) | 13 (34.2) | 62 (81.5) | 14 (18.4) |
Mixed (n = 87)      | 52 (59.8) | 35 (40.2) | 132 (75.9) | 42 (24.1) |

Mental status (n = 93)c | 41 (56.9) | 31 (43.0) | 109 (75.6) | 35 (24.3) |
Normal (n = 72)      | 14 (66.6) | 7 (33.3)  | 34 (80.9)  | 8 (19.0)  |
Impaired (n = 21)     | 14 (66.6) | 7 (33.3)  | 34 (80.9)  | 8 (19.0)  |

aFrequency compared with that of controls for A0/A0 genotype ($\chi^2 3 \times 2 = 7.78; p = 0.020$) and for A0 allele ($\chi^2 3 \times 2 = 7.78; p = 0.015$).
bFrequency compared with controls for A0/A0 genotype ($\chi^2 2 \times 2 = 4.208; p = 0.04$) and for A0 allele ($\chi^2 2 \times 2 = 5.197; p = 0.022$).
cPatients with disease duration ≥10 years.

PD = Parkinson’s disease.
frontotemporal dementia with parkinsonism linked to chromosome 17, where different missense and splice site mutations have been described. Some cases with documented tau gene mutations have been described without tau deposits. Thus, the absence of tau deposits in most PD cases is not sufficient to rule out the potential implications of tau protein in PD. The detected association may be a result of the presence of a nearby change to the intronic tau polymorphism in PD patients; similarly, it has been postulated to occur in PSP.

The possibility is now open to further analyze the tau gene in another series of patients with PD and to perform functional studies on the expression of tau protein to clarify the specific mechanism through which the tau gene could be involved in the pathogenesis of some PD cases.

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References

Major Depression Is a Risk Factor for Seizures in Older Adults

Dale C. Hesdorffer, PhD,*†† W. Allen Hauser, MD,*††§ John F. Annegers, PhD,§ and Gregory Cascino, MD

We tested the hypothesis that major depression meeting DSM-III-R criteria or medical therapies for depression increase the risk for unprovoked seizures. Major depression was associated with a sixfold increased risk for unprovoked seizures (95% CI, 1.56–22). The risk remained increased even when controlling for age, sex, length of medical follow-up, and medical therapies for depression. In the absence of known prior neurological insult, major depression is associated with an increased risk for unprovoked seizures.

Hesdorffer DC, Hauser WA, Annegers JF, Cascino G. Major depression is a risk factor for seizures in older adults. Ann Neurol 2000;47:246–249

Several studies suggest that depression is more common than expected among people with epilepsy.1–9 Most studies are cross-sectional and cannot address the time order of the association. The one prior published epidemiological study undertaken to evaluate the temporal association found that a history of depression increased the risk for developing unprovoked seizures in adults.7 The definition of depression was not standardized, and subjects were interviewed 4 to 6 weeks after their seizure onset but asked about depression in the past 6 months, making time order between depression and seizures uncertain.

We undertook a case-control study of newly diagnosed idiopathic/cryptogenic seizures in older adults in part to determine if depression diagnosed according to the DSM-III-R criteria was a risk factor for a first unprovoked seizure.

Methods

Cases

From a larger cohort,10,11 we selected the incidence cases of first unprovoked seizure at the age of 55 years and over first diagnosed between 1955 and 1984 in residents of Rochester, Minnesota. We excluded cases if their unprovoked seizure was preceded by established risk factors for epilepsy (clinically detected cerebrovascular disease, central nervous system infection, brain trauma associated with at least 30 minutes of unconsciousness or posttraumatic amnesia, brain surgery, primary or metastatic central nervous system tumor, mental retardation, or cerebral palsy). The resulting group of cases consisted of patients with new-onset idiopathic/cryptogenic seizures.

Controls

For each case, 2 controls matched in age (±5 years), sex, residency, duration of care in the community, and medical evaluation during the year when the corresponding case’s incident seizure came to medical attention (the index date) were selected from the registration system of the facility at which the case was first identified. Potential controls were excluded if they experienced any of the above risk factors for epilepsy before the index date.

Exposure Data

We used a modified version of the structured clinical interview for DSM-III-R12 to ascertain symptoms of depression preceding the index date, reviewing all medical records in the records-linkage system of the Rochester Epidemiology Project.13 If a person had more than one episode of depression prior to the index date, we collected information on the most severe episode. Presence and duration of the following features were evaluated: depressed mood; diminished interest or pleasure; weight change; insomnia or hypersomnia; psychomotor agitation or retardation; fatigue or loss of energy; feelings of worthlessness or guilt; poor concentration, poor memory or indecisiveness; desire to harm oneself; suicide plan or fantasy; and hospitalization for depression. We also abstracted information on physical illnesses, medications, and major life events that could lead to depression. After separately categorizing individuals with depression attributable to one of the above causes, DSM-III-R14 diagnoses of major depression were made in the presence of at least five symptoms of depression for at least 2 weeks.

Several confounders were considered: the use of electroconvulsive shock therapy (ECT), the use of tricyclic antidepressants, and the use of phenothiazines. Dose and duration of these medical therapies were abstracted.

Generalized-onset and partial-onset seizures were classified by an epilepsy specialist (W.A.H.) from the seizure description recorded in the medical records according to the criteria of the International League Against Epilepsy.15

Statistical Analysis

Data were analyzed with SAS16 using conditional logistic regression for matched sets (SAS Institute, Inc, NC). Models were constructed for the whole population and separately for generalized-onset and partial-onset seizures. Statistical testing was done at the two-tailed level of 0.05.
Results

Many symptoms of depression were more common among cases compared with controls (Table 1). The tendency for cases to report more symptoms of depression than controls was not explained by more medical visits by cases (hence a greater likelihood of recording such symptoms) prior to the index date (mean number of visits for cases, 150.7; mean number of visits for controls, 152; \( p = 0.9 \)). Cases had idiopathic/cryptogenic seizures, lacked major illnesses requiring frequent and intensive medical visits prior to their seizures, and had the same opportunity as controls to report symptoms.

Major depression diagnosed according to DSM-III-R criteria was six times more common among cases \((n = 9; 6\%\) than controls \((n = 3, 1\%; p = 0.003\)). Cases were four times as likely as controls to experience at least five symptoms of depression for less than 2 weeks \((11\) patients and 5 controls; \( p = 0.005\)). There was no difference between cases and controls in depression with an organic cause \((3\) cases and 2 controls; not significant [NS]) and depression associated with a major life event \((4\) cases and 6 controls; NS). The time interval between the most severe episode of depression and the index date was shorter for cases than for controls for major depression and for at least five symptoms of major depression for less than 2 weeks (Table 2).

Medical treatments for depression \((tricyclic antidepressants, phenothiazines, and ECT)\) were more common among cases than controls (Table 3). Neverthe-

Table 1. Frequency of Symptoms of Depression and Hospitalization for Depression among 145 Cases and 290 Controls

<table>
<thead>
<tr>
<th>Symptom</th>
<th>No. of Cases</th>
<th>No. of Controls</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depressed mood</td>
<td>36 (25%)</td>
<td>34 (12%)</td>
<td>0.004</td>
</tr>
<tr>
<td>Loss of interest</td>
<td>12 (8%)</td>
<td>6 (2%)</td>
<td>0.005</td>
</tr>
<tr>
<td>Weight change ≥ 5%</td>
<td>19 (13%)</td>
<td>14 (5%)</td>
<td>0.01</td>
</tr>
<tr>
<td>Hypersomnia/insomnia</td>
<td>19 (13%)</td>
<td>21 (7%)</td>
<td>0.008</td>
</tr>
<tr>
<td>Psychomotor agitation/retardation</td>
<td>15 (10%)</td>
<td>8 (3%)</td>
<td>0.02</td>
</tr>
<tr>
<td>Fatigue/loss of energy</td>
<td>16 (11%)</td>
<td>13 (4%)</td>
<td>0.06</td>
</tr>
<tr>
<td>Worthlessness/guilt</td>
<td>7 (5%)</td>
<td>8 (3%)</td>
<td>0.1</td>
</tr>
<tr>
<td>Poor concentration/poor memory/</td>
<td>7 (5%)</td>
<td>5 (2%)</td>
<td>0.1</td>
</tr>
<tr>
<td>indecisive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desire to harm self</td>
<td>2 (1%)</td>
<td>1 (0.3%)</td>
<td>0.1</td>
</tr>
<tr>
<td>Suicide plan or fantasy</td>
<td>4 (3%)</td>
<td>2 (1%)</td>
<td>0.2</td>
</tr>
<tr>
<td>Hospitalized</td>
<td>12 (8%)</td>
<td>11 (4%)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Table 2. Median Time in Years between Most Severe Episode of Depression and Index Date

<table>
<thead>
<tr>
<th>Type of Depression</th>
<th>Median Time for Cases (IQR)</th>
<th>Median Time for Controls (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major depression</td>
<td>2.5 (1.4–9.1)</td>
<td>16.9 (4.9–30.1)</td>
</tr>
<tr>
<td>At least five symptoms for less than 2 weeks</td>
<td>2.5 (1.1–9.1)</td>
<td>29.2 (16.9–30.1)</td>
</tr>
</tbody>
</table>

IQR = interquartile range.

Discussion

Depression is a risk factor for unprovoked seizures in older adults. This may be the result of a common antecedent for both conditions.

Only two published studies have addressed the temporal relationship between depression and epilepsy. Both studies focused on depression preceding epilepsy. Neither defines depression. In a hospital-based case series of 51 patients with late-onset epilepsy, 16% had a history of depression prior to their initial seizure. Such a hospitalized group probably represents the more severe forms of epilepsy, and the study had no control group. An association between depression and newly diagnosed unprovoked seizures in cases between 17 and 74 years of age was reported in a population-based epidemiological study. Depression was seven times more...
common among cases than controls ($p = 0.003$). In cases with a “localized” seizure, depression was 17 times more common than among controls ($p = 0.02$).

We found that in older adults, DSM-III-R depression is associated with a fourfold increased risk for developing a first unprovoked seizure, adjusting for medical therapies for depression. This increased risk was more prominent among cases with partial-onset seizures. Major depression occurred closer to the index date in cases than in controls, suggesting that pathophysiology leading to depression may also lower seizure threshold.

Our study has several advantages over previous work. We considered only depression prior to the first unprovoked seizure, thus avoiding problems with time order and recall bias. We adapted the structured clinical interview for DSM-III-R to make a standardized diagnosis of DSM-III-R major depression. Finally, we adjusted for the confounding effects of medical therapies for depression.

We have evaluated medically recognized depression. This may explain why the prevalence of depression is less than half of that expected based on previous reports in the literature from community surveys. The effects of this information bias should be nondifferential, resulting in an underestimate of the association between major depression and seizures. One study comparing personal interview with medical record review for ascertainment of major depression found that people were more likely to recall past episodes of depression when they were currently depressed. This implies that even personal interview may have misclassified some people who had suffered depression as never having been depressed.

An association between ECT, tricyclic antidepressants, phenothiazines, and seizures has been reported. These reports are anecdotal, the level of risk is never discussed, and the underlying disorder requiring such medication is not taken into account. We demonstrate that the association between such therapies and seizures can be explained almost entirely by major depression.

The increased frequency of depression among people with epilepsy reported in cross-sectional studies may not be solely attributable to seizures or to antiseizure therapy. Rather, an underlying comorbidity or pathology common to epilepsy and depression may explain these associations. Further work on the time order of these comorbid conditions may illuminate mechanisms responsible for both.

This work was funded in part through grants from the Epilepsy Foundation of America, NINDS (NS-16308) to the University of Minnesota, NIH (MO1RR00645) to Columbia University, and NIA (AG 06786) to the Mayo Foundation.

Thanks to Dr Alexander Lucas for his invaluable assistance and to Patricia Perkins for her help with data abstraction.

### References

6. Dominian MA, Serafetinides EA, Dewhurst M. A follow-up

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### Table 3. Odds Ratios and 95% CIs for DSM-III-R Major Depression, Tricyclic Antidepressants, Phenothiazines, Electroconvulsive Shock Therapy, and Unprovoked Seizures

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of Cases</th>
<th>No. of Controls</th>
<th>Crude Odds Ratioa</th>
<th>95% CI</th>
<th>Adjusted Odds Ratiob</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major depression(^\text{c})</td>
<td>9</td>
<td>3</td>
<td>6.0</td>
<td>1.56–22.0</td>
<td>3.7</td>
<td>0.8–17.0</td>
</tr>
<tr>
<td>Tricyclic antidepressants(^\text{c})</td>
<td>18</td>
<td>17</td>
<td>2.2</td>
<td>1.1–4.5</td>
<td>1.6</td>
<td>0.8–3.5</td>
</tr>
<tr>
<td>Phenothiazines(^\text{c})</td>
<td>43</td>
<td>62</td>
<td>1.6</td>
<td>1.0–2.5</td>
<td>1.3</td>
<td>0.8–2.2</td>
</tr>
<tr>
<td>ECT(^\text{c})</td>
<td>7</td>
<td>3</td>
<td>4.7</td>
<td>1.2–18</td>
<td>1.5</td>
<td>0.3–7.5</td>
</tr>
<tr>
<td>At least 5 depressive symptoms for less than 2 weeks(^\text{c})</td>
<td>11</td>
<td>5</td>
<td>4.4</td>
<td>1.5–13.0</td>
<td>(\text{e})</td>
<td>(\text{e})</td>
</tr>
<tr>
<td>Triggered depression(^\text{c,d})</td>
<td>7</td>
<td>8</td>
<td>2.8</td>
<td>0.98–7.9</td>
<td>(\text{e})</td>
<td>(\text{e})</td>
</tr>
</tbody>
</table>

\(^\text{a}\)Conditional odds ratio adjusting for the matching variables (age, sex, and duration of medical care in the community).

\(^\text{b}\)Conditional odds ratio adjusting for the matching variables and each independent variable.

\(^\text{c}\)Any time before the index date.

\(^\text{d}\)Depression triggered by a major life event, drug, or illness.

\(^\text{e}\)Excluded from multivariate model.
Novel Leu723Pro Amyloid Precursor Protein Mutation Increases Amyloid β42(43) Peptide Levels and Induces Apoptosis

John B. J. Kwok, PhD,* Qiao-Xin Li, PhD,† Marianne Hallupp, BSc,* Scott Whyte, PhD,‡ David Ames, MD,§ Konrad Beyreuther, PhD,‖ Colin L. Masters, MD,† and Peter R. Schofield, PhD, DSc*

A novel missense mutation, Leu723Pro, in the amyloid precursor protein (APP) gene was discovered in an early-onset Alzheimer’s disease family. Expression of L723P mutant APP complementary DNA in CHO cells resulted in a 1.4- to 1.9-fold increased production of the 42(43)-amino acid length amyloid β peptide compared with the wild-type sequence and was capable of causing apoptosis. The mutation is predicted to alter the luminal transmembrane length and helical arrangement of the APP molecule and thus affect the γ-secretase cleavage site.


The presence of senile plaques is one of the key neuropathological feature of Alzheimer’s disease (AD). Senile plaques are extracellular deposits composed mainly of the 39 to 42(43)-amino acid length amyloid β peptide (Aβ), which is cleaved by a series of secretases from the amyloid precursor protein (APP) (Fig 1). Analyses of families with early-onset AD (EOAD) have identified missense mutations in the APP and presenilin genes. All EOAD mutations result in the relative overproduction of the 42(43)-amino acid iso-
form of Aβ (Aβ42). The London APP mutation (V717I) and mutant presenilin genes have also been shown to induce apoptosis when expressed in transfected cells. In the present study, we present a novel mutation (L723P) in the APP gene in an Australian EOAD pedigree and its effects on APP metabolism and cytotoxicity via apoptosis. A preliminary report of this work was made at the 6th International Conference on Alzheimer’s Disease and Related Disorders, Amsterdam, July 18 to 23, 1998.

Materials and Methods

Pedigree and Genetic Analysis
The Australian EOAD pedigree consisted of 3 generations in which presenile dementia was inherited in an autosomal dominant manner (see Fig 1a). The mean age of onset for the family is 56 years. The proband presented at the age of 45 years, with a 4- to 5-year history of cognitive decline. On initial review, formal neuropsychological testing revealed him to have a low average IQ, with marked impairment in new learning, short-term memory, planning, and organizational abilities. A computed tomography scan of the head showed mild to moderate cerebral atrophy, and results from routine biochemical review and lumbar puncture were normal. A diagnosis of probable Alzheimer’s type dementia was made according to NINCDS-ADRDA criteria. Over the following 2 to 3 years, he experienced more generalized cognitive decline, with persecutory delusions, hallucinations, and aggressive behavior. Four years after the initial review, he was mute and fully dependent in personal care. No neuropathological data are available for the affected members of the pedigree. Intronic primers were used to amplify Aβ sequences from genomic DNA of pedigree members by polymerase chain reaction (PCR). PCR products were sequentially cycled using the HhaI restriction enzyme digest assay. Each APP CHO clone was metabolically labeled with 35S-methionine (150 µCi/ml) and maintained in growth medium supplemented with 0.2–fold increase in production of Aβ42 in APP (L723P)-expressing clones compared with clones expressing wild-type APP cDNA (see Fig 2b). This result was comparable to the twofold elevation in Aβ42 levels that we observed in CHO cell lines constitutively expressing the London or Swedish mutation.

To control for possible cytotoxic effects of APP, the APP cDNA was placed under the control of a tetracycline-responsive promoter. Each clone was induced by exposure to low concentrations of tetracycline (0.2 µg/ml) for 24 hours, followed by 3 hours of metabolic labeling in the absence of tetracycline. Cell lysate from each clone was examined for the production of Aβ. Pooled results from induced TO.APP clones resulted in a significant 1.4 ± 0.2–fold increase in production of Aβ42 (p < 0.03; two-tailed Student’s t test) in three APP (L723P)-expressing clones compared

Expression and Analysis of APP Complementary DNA Constructs

The 751–amino acid APP cDNA was subcloned into vector, pRe/C MV (Invitrogen, Carlsbad, CA) to produce the pCMV.APP constructs. The 770–amino acid APP cDNA was subcloned into the inducible Tet-Off bidirectional vector, pBI-G (Clontech, Palo Alto, CA) to produce the pTO.APP constructs. The L723P mutation was introduced into the APP cDNA constructs by oligonucleotide-directed mutagenesis. CHO.K1 or CHO-AA8 cell lines (Clontech) were maintained in Ham’s F12/Dulbecco’s Modified Eagle Medium with 10% fetal calf serum (Gibco-BRL, Grand Island, NY). pCMV.APP constructs were transfected into CHO.K1 cells using calcium phosphate. pTO.APP constructs were transfected into CHO-AA8 cells using Transfast reagent (Promega) and maintained in growth medium supplemented with 4 µg/ml of tetracycline-HCl.

Each APP CHO clone was metabolically labeled with 35S-methionine (150 µCi/ml) as described previously. Relative levels of Aβ species in either the supernatant or cell lysate

(50 mM of Tris-HCl, pH 7.5, 150 mM of NaCl, 2 mM of EDTA, 1% Nonidet P40, and 2 mM of phenylmethanesulfonyl fluoride) were analyzed by immunoprecipitation with mouse monoclonal antibodies specific for either the 40–amino acid (G2-10) or 42(43)–amino acid (G2-11) Aβ isoform. The cleavage of genomic DNA into nucleosomal length multimers in apoptotic cells was visualized by gel electrophoresis. The level of apoptosis observed in each clone was quantified using the Cell Death Detection ELISA PLUS kit (Boehringer Mannheim, Mannheim, Germany).

Results

Missense Mutation in APP Gene

A novel C-to-T missense mutation (C2168T; see Fig 1b) that results in the substitution of a leucine for a proline residue at codon position 723 of the APP molecule (770–amino acid isoform; see Fig 1c) was detected in the proband of an Australian EOAD pedigree (see Fig 1). The mutation was not found in any of the living unaffected relatives or in 90 unrelated chromosomes derived from normal individuals or other AD patients or pedigrees. The proband and his unaffected brother were ApoE3/ApoE4 heterozygotes, which suggests that familial clustering of AD in this pedigree was not caused by ApoE.

Overproduction of Aβ42 in L723P Mutant CHO Cell Lines

CHO clones transfected with APP expression constructs containing either the L723P mutation or wild-type sequence were analyzed for Aβ production. An autoradiograph of metabolically labeled and immunoprecipitated Aβ from conditioned media shows the 4- and 3-kd bands corresponding to Aβ and p3, respectively (Fig 2a). Aβ42 levels are expressed as a ratio of Aβ42/Aβ40 to normalize the amount of protein loaded. Pooled results from the conditioned media of CMV.APP CHO lines revealed a 1.9 ± 0.8 (mean ± SEM)–fold increase in production of Aβ42 in APP (L723P)-expressing clones compared with clones expressing wild-type APP cDNA (see Fig 2b). This result was comparable to the twofold elevation in Aβ42 levels that we observed in CHO cell lines constitutively expressing the London or Swedish mutation.

To control for possible cytotoxic effects of APP, the APP cDNA was placed under the control of a tetracycline-responsive promoter. Each clone was induced by exposure to low concentrations of tetracycline (0.2 µg/ml) for 24 hours, followed by 3 hours of metabolic labeling in the absence of tetracycline. Cell lysate from each clone was examined for the production of Aβ. Pooled results from induced TO.APP clones resulted in a significant 1.4 ± 0.2–fold increase in production of Aβ42 (p < 0.03; two-tailed Student’s t test) in three APP (L723P)-expressing clones compared
with clones expressing wild-type cDNA (see Fig 2c). Thus, the L723P mutation causes elevated production of Aβ42 relative to Aβ40, and these results are in the same range (1.5–2.0-fold elevation) observed for APP mutations.\textsuperscript{17,18}

**Apoptosis in TO.APP CHO Cell Lines**

To verify that the TO.APP CHO clones were dying via the apoptotic pathway, cells were harvested and examined for the cleavage of genomic DNA into nucleosomal length fragments. The distinctive DNA “ladder” is present in CHO-AA8 cells exposed to 150 nM of staurosporine, a potent inducer of apoptosis, but not in untreated CHO-AA8 cells. The same DNA ladder is found in representative wild-type and L723P mutant TO.APP clones (Fig 3a). Low levels of apoptosis in cells expressing wild-type APP cDNA have been observed previously.\textsuperscript{6} Each clone was quantified for the level of apoptosis using enzyme-linked immunosorbent assay at set time points after the removal of tetracycline. Twenty-four hours after induction of the pooled TO.APP(L723P) clones, the level of apoptosis was increased threefold compared with the parental CHO cells and the wild-type TO.APP (wild-type) clonal lines (see Fig 3b). Thus, the L723P mutation is capable of inducing apoptosis in transfected CHO cells.

**Discussion**

We report a novel Leu723Pro mutation in the APP gene of an Australian EOAD pedigree. We have shown...
that the expression of the L723P mutation in CHO cell lines results in a 1.4- to 1.9-fold overproduction of Aβ42. The position of the L723P mutation is surprising in that it is located at the end of the putative α-helical transmembrane domain of APP (see Fig 1c). This has implications for the nature of the cleavage site of the γ-secretase in the APP molecule, because the L723P mutation is located eight residues (two helical turns) from the γ-secretase sites and is also considerably further downstream of the three other putative γ-secretase mutations: the Italian (V715M), Florida (I716V), and London (V717I) mutations (see Fig 1c).3,4

Two studies have defined the nature of the γ-secretase site.19,20 Mutation scan analysis of the α-helical transmembrane region around the γ-secretase site has shown that there was little sequence specificity requirement.19 It was postulated that alterations in amino acids on one side of the α-helix interfered with direct interaction between γ-secretase and APP.19 However, the L723P mutation is on the opposite side of the proposed γ-secretase–active α-helical site. One possible explanation for the observed effect of the L723P mutation on Aβ42 production may be the spe-
cific amino acid residue introduced by the mutation. The substitution of a proline for a leucine at the cytoplasmic transmembrane junction is predicted to result in a kink in the protein and may possibly alter the topology of the α-helix. The second study employed insertion or deletion mutations that altered the length of the APP transmembrane region. The mutant APP molecules indicated that the γ-secretase cleavage site was independent of the actual amino acid sequence and was determined by the position of the APP transmembrane domain relative to the cell membrane and γ-secretase. The location of the L723P mutation at the transmembrane junction may also alter the luminal length of the APP transmembrane region and would thus be expected to have an impact on γ-secretase activity. To clarify the nature of the γ-secretase site, further functional studies are needed.

Although the overproduction of Aβ42 appears to be the underlying biochemical pathway in AD etiology, it is possible that other neurodegenerative mechanisms such as apoptosis may play a role. Both the London APP mutation and several presenilin mutations induce apoptosis when expressed in transfected cells. Our analysis indicates that like other AD mutations, the L723P mutation is capable of inducing apoptosis. The future challenge will be to determine whether the two phenomena, namely, amyloid deposition and apoptosis, are independent or perhaps different steps in the same pathogenic pathway.

References
Prenatal Testing for Late Infantile Neuronal Ceroid Lipofuscinosis

Elizabeth Berry-Kravis, MD, PhD,* David E. Sleat, PhD,† Istvan Sohar, PhD,‡ Peter Meyer, MD,§ Robert Donnelly, PhD,§ and Peter Lobel, PhD†

Classic late infantile neuronal ceroid lipofuscinosis (LINCL) is a neurodegenerative disease in which autofluorescent “curvilinear” storage bodies accumulate in tissues from affected patients. Recently, the LINCL gene (CLN2) has been found to code for a pepstatin-insensitive lysosomal protease whose activity is deficient in LINCL specimens. We report the first 2 cases of successful prenatal testing for LINCL by using DNA and enzyme-based methods on amniocytes, and describe a new private mutation in one of the families analyzed. These approaches allow definitive prenatal diagnosis and represent a significant improvement over previous pathological methods.


The neuronal ceroid lipofuscinoses (NCLs) are a heterogeneous group of neurodegenerative disorders characterized by accumulation of autofluorescent ceroid-lipofuscin storage material in lysosomes of neurons and other cell types. These disorders are classified according to age at onset, specific pathology, and most recently, molecular analysis.1 Specific forms include infantile NCL (INCL; CLN1), classic late infantile NCL (LINCL; CLN2), juvenile NCL (JNCL; CLN3), adult NCL (CLN4), two variant forms of LINCL (vLINCL; CLN5 and CLN6), and other atypical forms.

Classic LINCL is characterized by myoclonic and major motor seizures, ataxia, retinal degeneration, and progressive mental and motor decline, with onset at 2 to 4 years of age. The disease is invariably fatal, with death typically between 8 and 15 years of age. The CLN2 gene deficient in LINCL has recently been identified and encodes a lysosomal protease.2 Different assays that estimate CLN2 protease activity reveal very reduced or absent activity in specimens from LINCL patients,2–5 thus allowing rapid diagnosis of disease. In addition, DNA-based methods have been developed that allow mutation scanning of the entire coding region and splice junction regions6 and have defined 24 mutations associated with the disease.7

Previously, prenatal testing for LINCL has been accomplished through electron microscopic examination of uncultured amniocytes for typical curvilinear bodies.8,9 This method is considered difficult by some because of the large amount of tissue debris in amniocyte samples, including irregular trilaminar membranes, which could lead to misinterpretation.8,10 Although there are reports of successful prenatal diagnosis of NCLs based on subcellular pathology,8–11 there have also been unsuccessful attempts at diagnosis.12 Electron microscopy of fetal skin biopsy has been used for prenatal diagnosis of LINCL, but this method carries a higher level of fetal risk, is less available, and the sample may be inadequate. Identification of the LINCL gene and its product has allowed development of definitive diagnostic methods. In this report, we describe application of these methods toward prenatal screening for LINCL by amniocentesis in two at-risk pregnancies.

Cases and Methods

Cases

CASE 1. The proband was born at term to unrelated parents of European and Native American background. Early development was normal. At 3.5 years she had a generalized tonic–clonic convulsion and an electroencephalogram showed background slowing and generalized epileptic discharges with photic stimulation. She subsequently developed refractory generalized tonic–clonic and myoclonic seizures, prominent valproate-induced tremor, ataxia, and cognitive and speech decline. Although seizures, tremor, and myoclonus improved after treatment with topiramate and clonazepam, motor and mental deterioration continued. Electron microscopy of a rectal biopsy at age 4.5 years showed curvilinear storage bodies typical of LINCL in myocytes and endothelial cells. Diagnosis of LINCL was confirmed by genetic and enzyme-based testing on the affected child and parents (see Results). Several months after this, the family presented with concerns regarding a very early (6 week) pregnancy, and amniocentesis at 16 weeks’ gestation was performed for prenatal diagnosis via CLN2 analysis.

CASE 2. The proband was born 2 weeks before term to first cousins of Turkish origin. Early development was normal. The patient developed seizures at age 3 years and was unable to ambulate independently by age 5. Electron microscopy
performed on a skin biopsy demonstrated curvilinear bodies typical of LINCL. The parents presented with a subsequent pregnancy and amniocentesis was performed at 16 weeks' gestation. There were inadequate numbers of amniocytes for electron microscopy. Fetoscopy and skin biopsy also failed to yield a diagnosis, because no eccrine sweat glands were found in the tissue. Finally, molecular analyses of CLN2 were performed on cultured fetal amniocytes and DNA samples from the parents and proband.

CLN2 Analysis

Amniocytes were cultured to confluence in Chang’s media with 10% fetal bovine serum or in Amniomax (GibcoBRL, MD). CLN2 DNA sequence at the site of the parental mutations was determined by dye-primer sequencing, using the previously described strategy. CLN2 protease activity in control and patient buffy coat leukocytes and in control and at-risk fetal amniocytes was determined by using fluorescent hemoglobin substrate following the conditions described previously.

Results

The proband in Case 1 was a compound heterozygote for the two most common CLN2 mutations, an intronic 3556G > C 3’ splice junction mutation and a nonsense 3670C > T Arg208 > Stop mutation (Fig, left). The father carried the splice junction mutation and the mother the Arg208 mutation. CLN2 protease activity was present in control leukocytes but was essentially absent in leukocytes from the proband (Table 1). As described previously, normalization of CLN2 protease activity to total acid protease activity markedly reduced the coefficient of variation of the controls (CLN2 protease, coefficient of variation = 28%; ratio, coefficient of variation = 12%) and, using this activity ratio, both the mother and father (obligate heterozygotes) had intermediate activity (see Table, assay 1).

The Arg208 mutation, but not the splice junction mutation, was identified in DNA isolated from amniocytes from the fetus at risk (see Fig). This indicates that

Table 1. Enzyme Activities in Buffy Coat Leukocytes from Family 1 and Control Subjects

<table>
<thead>
<tr>
<th>Sample</th>
<th>CLN2 Proteasea</th>
<th>Acid Proteasea</th>
<th>Ratiob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>643</td>
<td>984</td>
<td>0.65</td>
</tr>
<tr>
<td>Control 2</td>
<td>731</td>
<td>1,239</td>
<td>0.59</td>
</tr>
<tr>
<td>Control 3</td>
<td>1,071</td>
<td>2,095</td>
<td>0.51</td>
</tr>
<tr>
<td>Affected child</td>
<td>27</td>
<td>1,100</td>
<td>0.02</td>
</tr>
<tr>
<td>Mother</td>
<td>226</td>
<td>1,007</td>
<td>0.22</td>
</tr>
<tr>
<td>Father</td>
<td>557</td>
<td>1,988</td>
<td>0.28</td>
</tr>
<tr>
<td>Assay 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>600</td>
<td>1,243</td>
<td>0.48</td>
</tr>
<tr>
<td>Control 2</td>
<td>534</td>
<td>1,043</td>
<td>0.51</td>
</tr>
<tr>
<td>Control 3</td>
<td>370</td>
<td>693</td>
<td>0.53</td>
</tr>
<tr>
<td>Mother</td>
<td>733</td>
<td>2,855</td>
<td>0.26</td>
</tr>
<tr>
<td>Baby</td>
<td>434</td>
<td>1,465</td>
<td>0.30</td>
</tr>
</tbody>
</table>

aData are expressed as picomoles per hour per milligram (pmol/hr/mg).
bNote that CLN2 protease and acid protease activities were measured by using different substrate concentrations, and thus, the ratio is for normalization purposes only.

Assay 1 was conducted at the time of diagnosis of the proband. The mother was not pregnant at the time. All samples were collected and processed in parallel.

Assay 2 was completed 8 months after delivery of the at-risk infant. All samples were collected and processed in parallel. Controls were from different individuals than those analyzed in assay 1.

CLN2 = classic late infantile neuronal ceroid lipofuscinose.

Fig. Electrophoretograms depicting the regions of interest for Family 1 (left) and Family 2 (right).
the fetus will be a carrier of LINCL but not affected. CLN2 protease activity was present in seven control amniocyte cultures, but even after normalization to total acid protease, the coefficient of variation was high (CLN2 protease, coefficient of variation = 30%; ratio, coefficient of variation = 20%) (Table 2). Compared with controls, both the CLN2 protease activity and the normalized CLN2 protease activity of the at-risk amniocytes were reduced (29% and 48%, respectively). Postnatal testing of the at-risk infant confirmed that the CLN2 protease/total acid protease ratio fell in an intermediate range similar to the mother’s values, suggesting carrier status (see Table 1, assay 2). Sequence analysis of DNA from the infant revealed the same genotype found in amniocytes, confirming carrier status.

The proband in Case 2 was homozygous for a 3664C > T Arg206Cys mutation (see Fig, right). No other changes were detected from sequence analysis of the entire CLN2 coding sequence, the 5′ untranslated sequence, splice junctions, most introns, and part of the putative promoter region. The mutation was not detected in 126 apparently unrelated individuals (LINCL families, families with other neurological diseases, and normal subjects) analyzed previously. Each parent was heterozygous for the 3664C > T change (see Fig, right). Assuming an accurate diagnosis of LINCL (see Discussion), these findings are consistent with the 3664C > T mutation being causative or tightly linked with disease.

Table 2. Enzyme Activities in Cultured Amniocytes

<table>
<thead>
<tr>
<th>Sample</th>
<th>CLN2 Protease</th>
<th>Acid Protease</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1 fetus</td>
<td>86</td>
<td>1,379</td>
<td>0.06</td>
</tr>
<tr>
<td>Case 2 fetus</td>
<td>210</td>
<td>2,972</td>
<td>0.07</td>
</tr>
<tr>
<td>Controls (n = 7)</td>
<td>296 ± 88</td>
<td>2,453 ± 762</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>Control 1†</td>
<td>208</td>
<td>2,291</td>
<td>0.09</td>
</tr>
<tr>
<td>Control 2†</td>
<td>214</td>
<td>1,366</td>
<td>0.16</td>
</tr>
<tr>
<td>Control 3†</td>
<td>422</td>
<td>3,526</td>
<td>0.12</td>
</tr>
<tr>
<td>Control 4†</td>
<td>384</td>
<td>2,979</td>
<td>0.13</td>
</tr>
<tr>
<td>Control 5†</td>
<td>233</td>
<td>1,603</td>
<td>0.15</td>
</tr>
<tr>
<td>Control 6†</td>
<td>259</td>
<td>2,712</td>
<td>0.10</td>
</tr>
<tr>
<td>Control 7†</td>
<td>352</td>
<td>2,691</td>
<td>0.13</td>
</tr>
</tbody>
</table>

aData are expressed as picomoles per hour per milligram (pmol/hr/mg).

bNote that CLN2 protease and acid protease activities were measured by using different substrate concentrations, and thus, the ratio is for normalization purposes only.

c–d Cultures collected and assayed in parallel.

Table 2. Enzyme Activities in Cultured Amniocytes

CLN2 = classic late infantile neuronal ceroid lipofuscinosis.

The two different tests used in this study have distinct advantages. The DNA-based tests are definitive but often take more time than the enzyme assay, particularly if the familial mutations are not known beforehand and are not the most common disease alleles. Some mutations may not be detected with the current sequencing strategy. The enzyme-based assay has the advantage that no previous knowledge of the underlying mutation is required. However, there is some variability in activity of normal controls, and although it is quite likely that the activity of affected amniocytes would be strikingly low compared with controls, this has not been directly demonstrated, because both at-risk fetuses had functional CLN2 genes. Thus, it will be important to continue to use both DNA and enzyme-based tests for prenatal diagnosis.

In Case 2, clinical data and electron microscopy were essentially diagnostic of LINCL, and the DNA analysis was completely consistent with this. However, the proband was not tested for CLN2 protease activity. Thus, there is a remote possibility that the apparent mutation actually represents a polymorphism that does not impair CLN2 gene function. If so, the family could carry an unlinked genetic lesion responsible for disease, and the DNA- and enzyme-based testing used here to exclude CLN2 defects would not be informative. Although this outcome seems improbable, when private mutations are detected by DNA-based methods, it is highly advisable to corroborate the diagnosis by enzyme-based testing.

In summary, the availability of direct genetic and enzyme-based testing for LINCL on cultured amnio-
cytes now allows rapid and definitive prenatal diagnosis. By using these methods, we were able to rule out disease in two at-risk pregnancies.

This study was supported by NS-37918 to Peter Lobel.
We are grateful to Rosalie Gin for technical assistance.

References


Libyan Jews living in Israel have a prevalence of Creutzfeldt-Jakob disease (CJD) exceeding 70 times that reported in other populations, including other ethnic groups in Israel.1,2 This cluster, once thought to be caused by ingestion of lightly grilled sheep brain tainted with scrapie,3 is now known to be caused by an autosomal dominant mutation in the prion protein (PrP) gene (PRNP) that results in the substitution of lysine for glutamate at residue 200.4,5 Because of variability in the age at disease onset, we hypothesized that environmental factors were somehow necessary to produce disease in genetically susceptible individuals.6 Whether only genetic or additional environmental factors are involved, a greater load of disease genes could be expected to result in disease with younger onset, a more rapid course, or a distinct phenotypic profile.

We identified 70 Creutzfeldt-Jakob disease patients with the previously described E200K mutation in the prion protein gene. The purpose of this study was to define the clinical features of E200K homozygous patients (n = 5), compared with heterozygotes. We found a statistically significant younger age at disease onset for the homozygous patients, although the average age at onset in this group was still in midlife. Disease features were not statistically different in the two groups. Possible explanations are discussed.


From the *Departments of Neurology, Tel-Aviv Sourasky Medical Center and Tel-Aviv University Sackler Faculty of Medicine, Tel Aviv; †Departments of Neurology, Barzilai Medical Center, Ashkelon; ‡Department of Neurology and Neuroepidemiology, Hebrew University Faculty of Medicine; and Centre de Recherche Français de Jerusalem, CNRS, Jerusalem, Israel.

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Address correspondence to Prof Korczyn, Sieratzki Chair of Neurology, Tel-Aviv University Medical School, Ramat Aviv 69978, Israel.
Since the late 1960s, we have monitored all identified cases of CJD in Israel. As part of this epidemiological survey, we identified 5 Libyan CJD patients homozygous for the E200K PRNP mutation. The purpose of the present study was to compare clinical features between the homozygous and heterozygous patient groups.

Patients and Methods

The study population consisted of the Jewish patients of Libyan origin who suffered from CJD during the years 1968 to 1996 and whose banked blood or tissue samples were tested for mutations of the PrP gene. Data regarding several of the patients were previously reported.5–11 Data were gathered from medical records, death certificates, electroencephalograms, and pathology reports. Age at onset was determined by the patient’s first presentation to medical care with any disease component. Disease components were classified as either present or absent, as recorded in the available records. When present, a symptom or sign was coded as either a presenting feature or as a late development in the disease course.

Testing for the E200K mutation in the PrP gene was performed by using standard techniques.4,5 DNA was usually extracted from blood samples, and a 717-bp sequence containing the PRNP gene was amplified by polymerase chain reaction. The polymerase chain reaction fragments were digested by the restriction enzyme BsmAI and the resulting products visualized by ethidium bromide staining in agarose gels.4,12,13

Descriptive statistics in the text are expressed as mean ± SD values. Statistical comparisons were performed to examine the null hypothesis that the clinical features of the homozygous patients were the same as those of the heterozygous cases with CJD. For continuous variables, such as the age at disease onset, the Wilcoxon test was performed. For nominal variables used to represent the presence of individual clinical features, χ² test was applied. Differences at the 5% level were considered significant. All statistics were performed by using SPSS statistical software (SPSS, Inc, Chicago, IL).

The individual clinical features were analyzed as distinct variables. In addition, new variables were computed for the combination of multiple signs within categories of clinical features (Table 1). Using logistic regression, we tested whether specific features tended to occur early in the development of the disease in one group compared with the other.

Results

Of the 70 patients, 65 patients (40 females) were heterozygous and 5 (2 females) were homozygous for the PRNP E200K mutation. Sixty-five patients were from Libya, Tunisia (or were born in Israel to Libyan parents), 3 were from Turkey, and 2 were from Egypt. There was a significantly younger age at disease onset for homozygous patients (see Table 1). Homozygous patients had longer duration of disease; however, this difference was not statistically significant. It is noteworthy that 1 of the homozygous patients (Table 2, Case 3) had a slowly progressive atypical course, with unique histochemical features (no protease-resistant PrP was present in the brain sample) and a disease duration of 96 months. It is possible that he had a novel disease form. Excluding him from the statistics, there was still a significantly younger age at onset in the homozygous patients (50.3 ± 8.9 vs 59.1 ± 9.0 years; p = 0.045), and disease duration was not significantly different between the groups (15.8 ± 15.9 vs 7.2 ± 6.3 months).

The distribution of the clinical signs is shown in Table 3. The disease feature was considered present if it was exhibited at all during the course of the disease. There were no significant differences in the prevalence of any of the disease features between the two groups, whether the analysis included or excluded Case 3. There were no distinguishing presenting symptoms be-

Table 1. Demographic Features of E200K CJD Patients

<table>
<thead>
<tr>
<th></th>
<th>Homozygote</th>
<th>Heterozygote</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>5</td>
<td>65</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>3/2</td>
<td>25/40</td>
</tr>
<tr>
<td>Age at onset, yr</td>
<td>50.4 ± 6.2</td>
<td>59.1 ± 9.0 (p = 0.03)</td>
</tr>
<tr>
<td>Range</td>
<td>41–58</td>
<td>35–77</td>
</tr>
<tr>
<td>Disease duration,* mo</td>
<td>15.8 ± 15.9</td>
<td>7.2 ± 6.3 (NS)</td>
</tr>
<tr>
<td>Range</td>
<td>2–96</td>
<td>2–28</td>
</tr>
</tbody>
</table>

*Excluding Case 3 (see explanation in text).

CJD = Creutzfeldt-Jakob disease.

Table 2. Details of Homozygous E200K CJD Cases

<table>
<thead>
<tr>
<th></th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
<th>Case 4</th>
<th>Case 5*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at onset, yr</td>
<td>49</td>
<td>41</td>
<td>51</td>
<td>49</td>
<td>53</td>
</tr>
<tr>
<td>Disease duration, mo</td>
<td>24</td>
<td>15</td>
<td>96</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Laboratory support for CJD</td>
<td>EEG</td>
<td>EEG</td>
<td>Biopsy</td>
<td>EEG</td>
<td>Autopsy</td>
</tr>
</tbody>
</table>

*Homozygosity in this patient was determined indirectly from genetic analysis of surviving family members, as follows. The patient’s wife tested negative for the E200K mutation, and all of their six children were verified as heterozygotes for the E200K mutation. Because the probability for this to be a chance occurrence is 1:64 had the patient been heterozygous, there is a high degree of certainty that the patient was, in fact, homozygous for the E200K mutation.

bUnlike all E200K patients previously tested, no protease-resistant PrP was detected (tested by western blot using anti-PrP mAb 3F4).

CJD = Creutzfeldt-Jakob disease; EEG = electroencephalogram; PrP = prion protein; mAb = monoclonal antibody.
between the two patient groups when clinical features were grouped into categories, as tested by logistic regression.

**Discussion**

We previously reported on the heterogeneity of CJD among carriers of the PRNP E200K mutation.9 There are no unique clinical features distinguishing patients with the E200K mutation from those with sporadic CJD.8 As an extension of these studies, we now examined the differences between Libyan CJD patients homozygous and heterozygous for the PRNP E200K mutation.7 There was a slightly earlier age at disease onset compared with heterozygous patients. However, none of the homozygous patients succumbed to the disease at a very young age and the disease phenotype was quite similar between the two groups.

Our patient series is unique, because CJD is a rare disease in which homozygosity for the E200K mutation is an even rarer occurrence. However, the results concerning the prevalence of clinical features must be interpreted with caution. First, the small number of patients in the homozygous group decreases the power of the study. There were many clinical features that failed to show statistical significance, despite seemingly large differences in frequency. It is possible that certain clinical features would be found statistically different in a larger group (type 2 error). Moreover, we could not determine exactly when a clinical feature appeared. The longer mean disease duration among homozygotes did not reach statistical significance but may be responsible for the recording of more signs. A second reason for caution relates to the retrospective nature of the data collection. The presence of a clinical feature could only be detected if it was positively noted in the patient record, and it is possible that there was nonuniform recording. We assume that the most obvious clinical signs were recorded, and there is no reason to suspect a bias in the reporting tendencies of clinical features with respect to the disease genotype.

Libyan CJD patients have an earlier age at onset than do sporadic CJD patients.8 The current finding of an even younger age at onset for homozygous CJD patients can be explained by a dose effect contributing to the onset of symptoms. It has indeed been shown that in CJD E200K patients, only the mutant and not the wild-type PrP produces protease-resistant PrP (PrPSc).14 The conversion of E200K PrP C → E200K PrPSc is assumed to be spontaneous, because no infectious step seems to be required to start the disease. A larger number of E200K PrP molecules in the homozygous patient’s brain may increase the chance of spontaneous conversion. It is noteworthy, however, that no homozygous patient came down with the disease as a teenager or a young adult.

Another possible explanation for the earlier disease onset in homozygous patients is that the absence of wild-type PrP may contribute to disease expression. This possibility is supported by results obtained from work with transgenic mice expressing human PrP proteins, which suggests that endogenous PRNP prevents the propagation of human prion disease in these animals.15 It has also been shown that humans carrying the E200K mutation are partially protected from contracting disease signs when wild-type PrP is present in excess.16 We further observed that homozygous patients may feature an unusual slowly progressive course without marked accumulation of PrPSc. This may suggest that the lack of wild-type PrP in homozygous patients could possibly have additive effects to those related to an absence of the normal PrP function. Such a possibility is also consistent with recent results in transgenic mice.17 According to this hypothesis, in some patients, such as Case 3 in our series, initial symptoms may be protracted and only later develop into a more typical CJD syndrome.

This study was supported in part by the Chief Scientist, Ministry of Health, Israel.
Vitamin E Deficiency due to Chylomicron Retention Disease in Marinesco-Sjögren Syndrome

Umbrerto Aguglia, MD,* Grazia Annesi, PhD,† Gianandrea Pasquinielli, MD,‡ Patrizia Spadafora, PhD,† Antonio Gambardella, MD,‖ Ferdinanda Annesi, PhD,† Angela Aurora Pasqua, PhD,† Francesca Cavalcanti, PhD,† Lucia Crecicbene, PhD,† Angelo Bagalà, PhD,† Francesco Bono, MD,* Rosario L. Oliveri, MD,‖ Paola Valentino, MD,* Mario Zappia, MD,* and Aldo Quattrone, MD‡

We report on 2 brothers (aged 19 and 12 years) with Marinesco-Sjögren syndrome who also had very low serum vitamin E concentrations with an absence of postprandial chylomicrons. The molecular study ruled out ataxia with isolated vitamin E deficiency, abetalipoproteinaemia, and hypobetalipoproteinaemia. The electron microscopy of the intestinal mucosa was consistent with a chylomicron retention disease. We speculate that both chylomicron retention disease and Marinesco-Sjögren syndrome are related to defects in a gene crucial for the assembly or secretation of the chylomicron particles, leading to very low serum levels of vitamin E.


The Marinesco-Sjögren syndrome (MSS) is a rare autosomal recessive form of severe cerebellar ataxia associated with congenital cataracts, mental deficiency, brisk tendon reflexes, skeletal anomalies, and cerebellar atrophy.1,2 The molecular and biological bases for MSS are unknown, and the diagnosis depends essentially on the clinical features. It is noteworthy that, although vitamin E deficiency is known to cause severe spinocer-
ebellar ataxia, serum vitamin E levels and lipid profiles have never been studied in MSS.

Here, we report the clinical, laboratory, ultrastructural, and genetic characteristics of 2 brothers with MSS, who were later shown to have a vitamin E deficiency related to chylomicron retention disease (CRD). The latter, also known as Anderson’s disease,3,4 is a rare autosomal recessive disorder characterized by a failure of the enterocytes to assemble or deliver lipoproteins leading to disturbed intestinal fat transport3,4 and severe vitamin E deficiency. As far as we are aware, this is the first report of an association between these two very rare disorders.

**Patients and Methods**

**Patients**
The family originated in a small village in Southern Italy. The parents were not consanguineous and had only 2 children, both with MSS. The parents and other relatives had no neurological disorders. An autosomal recessive mode of inheritance was likely.

The patients, 2 boys aged 19 (patient 1) and 12 (patient 2) years, were born at term after an uneventful pregnancy and delivery. On day 3 of life, Patient 1 had two isolated convulsions without hyperthermia. Cataracts were recognized early (between weeks 2 and 3 of age) and operated on at the age of 3 years in both subjects. During the newborn period, they were noted to have frequent episodes of diarrhea with steatorrhea and vomiting. They had malnutrition and failure to thrive. Later, they began to refuse milk and other fatty meals, preferring a protein-glucose diet. Their psychomotor development was severely delayed. Walking started at 7 (Patient 1) and 9 (Patient 2) years, and language development was poor with dysarthria. Complete sphencterine continence was acquired between the ages of 4 and 5 years. No cryptorchidism was found in either patient. Pubertal development was complete at the age of 17 years in Patient 1, and it was incomplete in Patient 2. At the time of neurological examination, both patients had severe cerebellar ataxia with truncal and limb ataxia, cerebellar dysarthria, nystagmus, strabismus without ophthalmoparesis, brisk tendon reflexes of the four limbs, flexor plantar responses, slightly decreased vibratory sensation in the lower limbs, weakness and atrophy of both proximal and distal muscles in the four limbs, low IQ (50) on the Wechsler Adult Intelligence Scale in Patient 1; 40 on the Wechsler Intelligence Scale for Children-Revised in Patient 2. Fundoscopy was normal in Patient 1, and revealed small areas of polar retinal depigmentation in Patient 2. A general examination disclosed pes planus, kyphoscoliosis, genu valga, and carinate chest. Abdominal ultrasonography was normal, and no involuntary movements, telangiectasia, or facial dysmorphism were found. Height was 168 cm (10th to 25th centile) and 148 cm (above the 50th centile), weight was 45 kg (below the 50th centile) and 38 kg (above the 75th centile) in Patients 1 and 2, respectively. Cerebrospinal fluid examination, performed in Patient 1, revealed a normal glucose and protein concentration; oligoclonal banding was absent.

Electrocardiographic, echocardiographic, and electroencephalographic (EEG) findings, auditory brainstem potentials, somatosensory evoked potentials, after stimulation of either the median or the peroneal nerve, were always normal, whereas the audiometric study showed a slight bilateral sensorineural hypoacusia in both patients. Visual pattern evoked potential recordings showed a delayed latency for the P100 wave (right eye = 160 msec, left eye = 131 msec, in Patient 1; right eye = 128 msec, left eye = 117.5 msec, in Patient 2; normal range = 89.5–111 msec). In both patients, computed eye movement analysis showed square wave jerks, slow saccade velocity, markedly decreased accuracy; reduced smooth pursuit gain, cogwheel pursuit, and gaze-evoked nystagmus. On needle electromyography, performed in Patient 1, no abnormalities were recorded from the abductor pollicis brevis, vastus medialis, tibialis anterior, and extensor digitorum brevis muscles. Nerve conduction studies showed markedly reduced sensory nerve conduction velocities for the sural nerves. A sural nerve biopsy, performed in Patient 1, showed a loss of large myelinated fibers without onion bulb formation. A biopsy of the peroneus brevis muscle, performed in Patient 1, showed signs of slight denervation. There were no ragged-red fibers. Staining with Oil Red O, periodic acid–Schiff, cytochrome c oxidase, NADH-TR, adenylyl deaminase, nonspecific esterase, alkaline phosphatase, and succinate dehydrogenase, as well as the immunohistochemical study with antidesmin antibodies produced normal results. On cranial magnetic resonance imaging scans, both patients had a dilated fourth ventricle, severe atrophy of the lower lobules of the cerebellar hemispheres and vermis, and preservation of the brainstem and cerebrum (Fig 1).

**Laboratory Findings**
The relevant laboratory findings are summarized in the Table.

The following laboratory findings were normal in both patients: serum levels of apolipoprotein-A1 (apo-A1), glucose, creatinine, urea, uric acid, total protein, albumin, α₁, α₂, β-, and γ-globulins, IgG, IgA, IgM, choline esterase, C-reactive protein, aldolase, γ-glutamyl transpeptidase, ceruloplasmin, α-fetoprotein, carciinoembryonic antigen, calcium, phosphate, magnesium, sodium, potassium, iron, transferrin, vitamin D, vitamin B₁₂, triiodothyronine (T₃), thyroxine (T₄), thyroid-stimulating hormone, growth hormone, parathyroid hormone; plasmatic levels of fibrinogen, lactate, pyruvate, pythanic acid, and very-long-chain fatty acids; analyses of amino acids in both plasma and urine; lysosomal enzymes in leukocytes (aryl-sulfatase A, α-glucosidase, β-glucosidase, β-galactosidase, α-galactosidase); complete blood count, erythrocyte sedimentation rate, prothrombin time, activated partial thromboplastin time; both standard urine and stool tests, and urinary concentrations of oligosaccharides and mucopolysaccharides. Both anti-β-gludin and anti-endomyosium antibodies were absent. Moreover, no abnormalities, such as acanthocytes or vacuolation in the lymphocytes, were discerned in the peripheral blood of either patient.

**Genetic Study**

**CYTOGENETIC STUDY.** The presence of spontaneous chromosomal abnormalities was examined in both patients by the...
Table. Relevant Laboratory Findings in Two Brothers with Marinesco-Sjögren Syndrome

<table>
<thead>
<tr>
<th>Examinations</th>
<th>Normal Range (On Fasting)</th>
<th>Patient 1 (On Fasting)</th>
<th>Patient 2 (On Fasting)</th>
<th>Patient 2 (After a Fatty Meal)</th>
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<tbody>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>140–200</td>
<td>80</td>
<td>94</td>
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<td>High-density lipoprotein cholesterol (mg/dl)</td>
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<tr>
<td>Very-low-density lipoprotein cholesterol (mg/dl)</td>
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<td>4.8</td>
<td>5</td>
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<tr>
<td>Apolipoprotein-A1 (mg/dl)</td>
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<td>130</td>
<td>147</td>
<td>115</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
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<td>83</td>
<td>113</td>
<td>123</td>
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<td>Chylomicrons</td>
<td>Absence</td>
<td>Absence</td>
<td>Absence</td>
<td>Absence</td>
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<td>Vitamin E (µmol/L)</td>
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<td>1</td>
<td>5</td>
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<tr>
<td>Vitamin B₁₂ (µg/ml)</td>
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<td>234</td>
<td>652</td>
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<tr>
<td>Folic acid (µg/ml)</td>
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<td>3.2</td>
<td>4.1</td>
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<td>Creatine kinase (U/L)</td>
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<td>285</td>
<td>204</td>
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<td>Aspartate aminotransferase (U/L)</td>
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<tr>
<td>Alanine aminotransferase (U/L)</td>
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<td>Lactate dehydrogenase (U/L)</td>
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<td>493</td>
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<td>Total bilirubin (mg/dl)</td>
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<td>2.4</td>
<td>1.5</td>
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<td>Direct bilirubin (mg/dl)</td>
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<td>0.4</td>
<td>0.3</td>
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<td>Ferritin (ng/ml)</td>
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<td>2</td>
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<tr>
<td>Follicle-stimulating hormone (mIU/ml)</td>
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<td>4.1</td>
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<td></td>
<td>Postpubescent = 1–9</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Luteinizing hormone (mIU/ml)</td>
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<td></td>
<td>3.2</td>
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<tr>
<td></td>
<td>Postpubescent = 1–5</td>
<td></td>
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</tbody>
</table>

Fig 1. Cranial magnetic resonance imaging scan of Patient 1. Note the dilated fourth ventricle, severe atrophy of the lower lobules of the cerebellar hemispheres and vermis, and the preservation of the brainstem and cerebrum.
G-banding method, using peripheral lymphocytes, and revealed no numerical or structural aberrations.

GENE ANALYSES. Genomic DNA was isolated from buffy coat previously prepared from whole blood that had been anticoagulated with EDTA. Gene analyses for spinocerebellar ataxia types 1, 2, 3, 6, and 7, for hereditary dentatorubropallidolusian atrophy and for Friedrich’s ataxia, excluded the diagnoses of these disorders. No mitochondrial DNA mutations of A3243G or A8344G were detected in either patient. Furthermore, the genotyping for both the α-tocopherol transfer protein and the apo-B genes was also performed in the patients and their parents. The linkage (MLINK software) of the family to both genes was excluded. Finally, we studied the microsomal triglyceride transfer protein gene in both patients by single-strand conformational polymorphism analysis. No abnormalities were found in exons 1 to 18 of the microsomal triglyceride transfer protein gene, which encompasses the entire coding region.

Ultrastructural Study of the Intestinal Mucosa
Duodenjejunal fragments were obtained by fiber optic endoscopy from Patient 1. Ultrastructural examination showed jejunal villi covered by normal looking and lipid-laden enterocytes. The latter had a patchy distribution and were easily recognized because of their collections of lipid vacuoles (Fig 2). Large and irregularly shaped vacuoles, mostly representing distorted and enlarged Golgi complexes, were found close to the cell nucleus. They contained multiple lipid droplets in the chylomicron-size range (100–1,000 nm in diameter). The microvilli were unremarkable. Intercellular membranes were juxtaposed and sealed by desmosomes; no lipid droplets were found within the intercellular spaces.

Discussion
The patients described here fulfilled the diagnostic hallmarks for MSS, including autosomal recessive inheritance, congenital cataracts, subnormal physical development, cerebellar ataxia, mental deficiency, brisk tendon reflexes, skeletal anomalies, and atrophy of the cerebellar hemispheres with a hypoplastic vermis on magnetic resonance imaging scans. As occasionally reported in patients with MSS, they also had optic neuropathy, hypergonadotropic hypogonadism, and a moderately elevated serum concentration of creatine kinase. Elevated serum concentrations of creatine kinase have also been found in mitochondrial myopathy presenting as atypical MSS. Nonetheless, both electromyographic and muscle biopsy studies failed to show any myopathic changes in our patients.

It is noteworthy that our patients also had very low concentrations of serum vitamin E, with a characteristic alteration of the lipid profile. These biochemical abnormalities were related to pathologically proven CRD, which is a very rare autosomal recessive disorder in which a failure of the enterocytes to assemble or deliver lipoproteins causes disturbed intestinal fat transport with a subsequent malabsorption syndrome. Subjects with CRD always have low serum concentrations of total, high-density lipoprotein and low-density lipoprotein cholesterol, and apo-B. Moreover, they have normal fasting concentrations of triglycerides and an absence of postprandial delivery of chylomicrons. Vitamin A and D concentrations may be normal or below the normal range, but vitamin E concentrations are dramatically affected or undetectable in all
patients. The genetic defect underlying CRD is still unknown. Clinically, the disease is characterized by severe diarrhea with steatorrhea in childhood, malnutrition, and failure to thrive. Mild neurological disturbances, including mental deficiency, disappearance of deep tendon reflexes, decreased vibratory sensation, axonal polyneuropathy, mild deficits of color perception, nystagmus, and action tremor may occur, but cerebellar ataxia has never been reported in CRD.

Of interest, both of our patients had normal serum concentrations of apo-A1 and vitamin D, despite very low serum concentrations of vitamin E and laboratory findings of mild liver impairment. A plausible explanation of this finding is the observation that chylomicron retention was not a diffuse finding on ultrastructural examination. In fact, we observed spared villi with normal looking enterocytes adjacent to others having lipid-laden enterocytes. Another interesting finding was that, although they had steatorrhea during early childhood, this was no longer observed. The preferred protein-glucose dietary regimen they adopted during childhood may reasonably explain the disappearance of steatorrhea in both patients.

In conclusion, the present study suggests that both CRD and MSS are related to defects in a gene that is crucial to the assembly or secretion of the chylomicron particle, leading to very low serum concentrations of vitamin E. Further studies are needed to determine precisely the link between these two very rare disorders.

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We thank Dr Carol Shoulders from the Molecular Medicine Group, MRC Clinical Sciences Centre, Imperial College School of Medicine, London, for the sequences of primers for analyzing the microsomal triglyceride transfer protein exons, and Dr Edoardo Lamberti-Castronuovo from the Istituto di Ricerche Cliniche “De Blasi,” Reggio Calabria, and Dr Agostino Gnasso from the Departimento di Medicina Sperimentale e Clinica, University of Catanzaro, for the study of lipid metabolism.

References
Genetic and Neuroradiological Heterogeneity of Double Cortex Syndrome

Joseph G. Gleeson, MD,*†‡ Robert F. Luo,* P. Ellen Grant, MD,‡ Renzo Guerrini, MD,§ Peter R. Huttenlocher, MD,§ Michel J. Berg, MD,¶ Stefano Ricci, MD,¶ Raffaella Cusmai, MD,,** James W. Wheelless, MD,†‡ Samuel Berkovic, MBBS,‡‡ Ingrid Scheffer, MBBS,‡‡ William B. Dobyns, MD,§§ and Christopher A. Walsh, MD, PhD*‡‡

From the *Division of Neurogenetics, Department of Neurology, Beth Israel Deaconess Medical Center, and †Programs in Neurosciences and Biological and Biomedical Sciences, Harvard Medical School, Harvard Institutes of Medicine, ‡Department of Neurology, Children’s Hospital, and ¶Department of Radiology, Massachusetts General Hospital, Boston, MA; §Institute of Child Neurology and Psychiatry, University of Pisa, IRCCS Stella Maris Foundation, Cambrone, and #Department of Neurological Sciences, Epilepsy Center, University of Rome “La Sapienza,” and **Ospedale Pediatrico Bambino Gesù, Rome, Italy; Departments of †Pediatrics and Neurology, and §§Human Genetics, University of Chicago, Chicago, IL; ‡Department of Neurology, University of Rochester Medical Center, Rochester, NY; ††University of Texas Medical School at Houston, Texas Comprehensive Epilepsy Program, Houston, TX; and ‡‡University of Melbourne, Austin and Repatriation Medical Centre, Heidelberg, Australia.

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Address correspondence to Dr Walsh, Division of Neurogenetics, Beth Israel Deaconess Medical Center/Harvard Medical School, Harvard Institutes of Medicine, 77 Avenue Louis Pasteur, Boston, MA 02115.

#Present address: Department of Neurosciences, Division of Pediatric Neurology, University of California, San Diego, School of Medicine, 9500 Gilman Dr, La Jolla, CA 92037-0624.

In the “double cortex” syndrome (DC; also known as subcortical laminar heterotopia or subcortical band heterotopia) and X-linked lissencephaly (XLIS), neurons leaving the ventricular zone during development fail to reach their final destination in the cortex, leading to epilepsy and mental retardation. DC is thought to occur when migration of a subset of cortical neurons arrests before completion to form a “double” cortex, a band of gray matter located within the subcortical white matter.1,2 XLIS results from the failure of proper migration of cortical neurons, leading to a much more severe disorder of cortex development.3 Characterization of several pedigrees with DC in females and lissencephaly in males identified a novel gene, doublecortin (DCX = gene; DCX = protein), in Xq22.3-q24 that was responsible for DC/XLIS.4,5 Previous studies have differed widely in DCX mutation identification rate. We found mutations in the DCX coding region in 8 of 8 pedigrees (100%) and 18 of 47 (38%) sporadic DC patients,6 whereas another study found mutations in 10 of 11 (91%) sporadic DC patients.7

This study was undertaken to better assess the role of DCX in a large sample of sporadic DC cases, to evaluate the sensitivity of single-strand conformational polymorphism (SSCP) analysis as a method for DCX mutation detection, and to determine if clinical features or magnetic resonance imaging (MRI) scan appearance could differentiate patients with a DCX mutation from those without a DCX mutation. We hypothesized that perhaps DC represents a heterogeneous genetic and possibly phenotypic group of diseases. To complete a comprehensive mutation analysis, the entire open reading frame of DCX was directly sequenced in all patients without a previously identified mutation (n = 16). In addition, brain MRI scans and clinical data were analyzed to see if any abnormalities could explain the genetic differences in our patients.

Patients and Methods

Patients

Thirty-one sporadic female patients with a referring diagnosis of DC were included in this study, comprising the Boston cohort of patients. Patients were enrolled according to clinical protocols defined by the Institution Review Boards at Beth Israel Deaconess Medical Center, Children’s Hospital, Boston, and other participating medical centers.

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Table 1. Clinical Data

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Mutation</th>
<th>Age (yr)</th>
<th>Age at Seizure Onset</th>
<th>Seizure Description</th>
<th>Seizure Severity</th>
<th>IQ/Mental Retardation</th>
<th>MRI Scan DC Category</th>
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<td>7 mo</td>
<td>Myoclonic</td>
<td>4</td>
<td>Borderline</td>
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</tr>
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<td>CPS</td>
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<td>17</td>
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<td>4</td>
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<td>4</td>
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<td>13</td>
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<td>CPS, GTCS, LGS</td>
<td>4</td>
<td>IQ 25</td>
<td>Posterior</td>
</tr>
<tr>
<td>39</td>
<td>No</td>
<td>22</td>
<td>2 yr</td>
<td>CPS</td>
<td>3</td>
<td>IQ 64</td>
<td>Limited/unilateral</td>
</tr>
<tr>
<td>40</td>
<td>No</td>
<td>46</td>
<td>15 yr</td>
<td>CPS</td>
<td>3</td>
<td>Borderline</td>
<td>Anterior/global</td>
</tr>
<tr>
<td>43</td>
<td>No</td>
<td>12</td>
<td>6 mo</td>
<td>CPS, GTCS, LGS</td>
<td>4</td>
<td>Mod-prof</td>
<td>Posterior</td>
</tr>
</tbody>
</table>

Patient numbers refer to those used previously. Patients had either no mutation in DCX, a mutation resulting in amino acid substitution, or a mutation causing protein termination. Amino acid substitutions indicate the original amino acid (aa), the new amino acid, and the amino acid number (R = arginine; H = histidine; I = isoleucine; T = threonine; N = asparagine; K = lysine; P = proline; V = valine). A protein termination is indicated by a "stop" at the corresponding amino acid. Magnetic resonance imaging (MRI) scan category indicates to which of the three radiographic groups, as follows, each patient was assigned: anterior biased/global double cortex (DC), posterior biased DC, and limited/unilateral DC. Age at seizure onset is indicated in years (yr) or months (mo). Seizure descriptions are as follows: GTCS = generalized tonic/clonic seizures; LGS = Lennox-Gastaut syndrome; CPS = complex partial seizures, myoclonic, atomic, and atypical absence. Seizure severity rating and IQ/mental retardation categories are detailed in the Patients and Methods section. Note that all patients with mutation have anterior biased/global DC, whereas none of the patients with posterior biased DC or limited/unilateral DC category were found to harbor DCX mutations.

Mutation Analysis
Genomic DNA isolation and polymerase chain reaction were performed as previously described. All patients without a previously identified mutation based on SSCP and Southern analysis (16 of 31) underwent direct sequencing analysis of the entire DCX open reading frame covering the splice sites. Each polymerase chain reaction product was sequenced in both directions following standard BigDye Terminator chemistry with an ABI 377 automated DNA sequencer (PE Biosystems, Foster City, CA). Direct sequence analysis allowed for either identification or exclusion of genetic heterozygosity.

Clinical Data and Brain MRI Study
Clinical information was obtained from referring physicians for all but 1 patient. Age at seizure onset, seizure type, seizure severity, and level of cognitive function were compared across all patients. For statistical analysis, patients were classified by seizure type into one of the following four categories: 1 = focal seizures (complex partial); 2 = myoclonic, atomic, or atypical absence seizures; 3 = generalized convulsive (tonic/clonic) seizures; and 4 = either multiple seizure types or Lennox-Gastaut syndrome. Seizure severity was based on the following scale: 1 = no seizures or seizures in remission without surgery; 2 = no seizures when treated with one or more drugs; 3 = partially drug resistant (seizure frequency, ≤1/mo when treated with one or more drugs); and 4 = intractable seizures (seizure frequency, >1/mo when treated with one or more drugs or a history of seizure surgery). Level of cognitive function was graded based on IQ, and the following categories were used: normal = IQ of
Patients were categorized into one of three groups based on magnetic resonance imaging (MRI) scan appearance. Band appearance, pachygyria/agyria characteristics, degree of malformation, and ventricle appearance were used to differentiate between these groups. The anterior biased/global double cortex (DC) category is composed of two groups, ie, one with DCX mutations and one without DCX mutations. None of the patients in the posterior biased DC category or limited/unilateral DC category were found to harbor DCX mutations.

<table>
<thead>
<tr>
<th>MRI Characteristics</th>
<th>Anterior Biased or Global DC</th>
<th>Posterior Biased DC</th>
<th>Limited or Unilateral DC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band appearance</td>
<td>Predominantly anterior or global, bilateral</td>
<td>Predominantly posterior, bilateral</td>
<td>Incomplete or unilateral band</td>
</tr>
<tr>
<td>Pachygyria/agyria characteristics</td>
<td>More severe anterior or symmetric</td>
<td>More severe posterior</td>
<td>None to mild</td>
</tr>
<tr>
<td>Degree of malformation</td>
<td>Mild to severe</td>
<td>Predominantly severe</td>
<td>Mild</td>
</tr>
<tr>
<td>Ventricle appearance</td>
<td>Normal or ventriculomegaly</td>
<td>Normal, trigonencephaly, or ventriculomegaly</td>
<td>Normal, trigonencephaly, or ventriculomegaly</td>
</tr>
</tbody>
</table>

Patients were categorized into one of three groups based on magnetic resonance imaging (MRI) scan appearance. Band appearance, pachygyria/agyria characteristics, degree of malformation, and ventricle appearance were used to differentiate between these groups. The anterior biased/global double cortex (DC) category is composed of two groups, ie, one with DCX mutations and one without DCX mutations. None of the patients in the posterior biased DC category or limited/unilateral DC category were found to harbor DCX mutations.

Results

Mutation Analysis

Direct sequencing of all coding exons identified only one mutation not detected by SSCP. Patient 26 displayed a C-to-T mutation at base 115, creating a nonsense stop codon with protein termination at amino acid 39. Of the 31 females examined in this study, 15 (48%) still lacked mutations after completion of mutation analysis.

Clinical Data and Brain MRI Study

A comparison of clinical data across patients can be seen in Table 1. No significant differences were found in age at seizure onset, seizure type, seizure severity, or level of cognitive function between patients with DCX mutations and those without mutations ($p > 0.05$).

Discussion

The results of this study suggest that DC may represent a genetically heterogeneous group of diseases and may not all be the result of germline mutations in DCX, as was previously hypothesized. Approximately 50% (16 of 30) of DC patients in our nonselected cohort displayed an identifiable DCX mutation, based on careful SSCP and direct sequencing of all coding regions and splice sites. It is noteworthy that the MRI scans from patients with an identifiable DCX mutation are remarkably similar, with an anterior biased subcortical band and overlying pachygyria, whereas some patients without identifiable mutations have posterior biased characteristics. This suggests that genetic heterogeneity may exist for DC. Curiously, approximately 25% (8 of 30) of patients in our cohort had scans that we could not distinguish from those with mutations, yet we could find no mutations in these patients. Several potential explanations have been proposed for the absence of identifiable mutations among patients with DC, and it remains to be seen if other types of DCX mutations are present.
mutations, perhaps in the large 3’ untranslated region, in the promoter region, in an undetected splicing mutation, or in a mutation within an intron, underlie DC in these cases.

We hypothesize that mosaicism at the DCX locus may account for some cases of DC in which no mutation was identified. In this instance, a de novo mutation in DCX would be present in only a portion of cells in the body and may not be detectable through DNA analysis from peripheral blood lymphocytes. Supporting this hypothesis is the finding that 3 mutation-negative patients in our cohort display limited/unilateral DC, which is the phenotype one would expect with a mosaic mutation. If mosaic mutations in DCX are found to underlie a significant fraction of DC, one could hypothesize that perhaps even more subtle forms of focal cortical dysplasias may be caused by mosaicism at the DCX locus.

Fig. Brain magnetic resonance imaging scans from 16 representative patients included in this study. The four rows are as follows: anterior biased/global double cortex (DC) with DCX mutation, anterior biased/global DC without mutation, and posterior biased DC without mutation, limited/unilateral DC without mutation. Anterior biased/global DC scans contained a bilateral band and pachygyria, with imaging abnormalities that were either symmetric or more severe anteriorly. Posterior biased DC scans were characterized by more severe posterior band and cortical malformations. Limited/unilateral scans showed either incomplete or one-sided bands, with none to mild pachygyria/agyria. Arrows indicate location of band. For Patient 39, a coronal image has been used because the band is more easily seen here than in an axial cut. Numbers in upper left corner of scans indicate patient number.
The present findings suggest that posterior biased DC may also not be the result of DCX mutations, because none of these patients displayed a DCX mutation. Although the absence of an identifiable DCX mutation does not preclude a role for DCX in these cases, it seems unlikely given their characteristically distinct radiographic appearance. Recent findings suggest that mutations in a lissencephaly gene, possibly LIS1 or an unidentified gene, may be responsible for some cases of DC with a posterior predominance. Pilz and colleagues found that lissencephaly patients with more severe imaging abnormalities anteriorly were more likely to display DCX mutations, whereas patients with more severe imaging abnormalities posteriorly were more likely to display LIS1 mutations. In accord with these recent findings, the results of the current study suggest that DC patients with an anterior bias are more likely to display DCX mutations. Last, the lack of significant differences in clinical data across all patients suggests that DC covers a wide range of band and gyral malformations with similar clinical manifestations. Physicians currently encounter a broad spectrum of DC cases, and important distinctions should be made among the different forms of DC.

This study was supported by grants from the National Institute of Neurological Diseases and Stroke (NINDS) (RO1-NS35129 to C.A.W. and RO1-NS35515 to W.B.D.), by the Human Frontier Science Program (to C.A.W.), and by the Mental Retardation Research Center at Children’s Hospital. Dr Gleeson was supported by a Neurological Sciences Academic Development Award fellowship (SK12NS01701-04) from the NINDS, from a National Epifellows Award from Parke Davis, and from an educational grant from Sigma Tau Pharmaceuticals. Dr Walsh is a scholar of the Rita Allen Foundation.

We express our sincere thanks to the patients who participated in these studies and to the clinicians who provided patient samples not directly used for this work.

References

Increased Numbers of CCR5+ Interferon-γ– and Tumor Necrosis Factor-α–Secreting T Lymphocytes in Multiple Sclerosis Patients

Tobias Strunk,* Stefanie Bubel, MD,† Beate Mascher,* Peter Schlenke, MD,* Holger Kirchner, MD,* and Klaus-Peter Wandinger, MD‡

To determine the frequency of in vivo activated T H1 lymphocytes, T-cell subsets of 9 multiple sclerosis patients with active disease and 17 healthy controls were analyzed by immunostaining for CCR5, CD26, and their expression of interleukin-2, interferon-γ, and tumor necrosis factor-α. The numbers of CCR5+ interferon-γ– and tumor necrosis factor-α–producing T cells were significantly increased in the peripheral blood of multiple sclerosis patients. CCR5 expression may be a useful marker to identify effector cells in multiple sclerosis and could be used as a tool for monitoring disease activity.


From the *Institute of Immunology and Transfusion Medicine, †Department of Neurology, University of Lübeck School of Medicine, Lübeck, Germany; and ‡Neuroimmunology Branch, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, MD.

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Address correspondence to Dr Wandinger, Neuroimmunology Branch, NINDS, National Institutes of Health, Building 10 Room 5B-16, 10 Center DR MSC 1400, Bethesda, MD 20892-1400.
Multiple sclerosis (MS) is believed to be an autoimmune disorder of the central nervous system white matter that is mediated by myelin protein-reactive T cells. The cause of the disease is unknown. However, activation of T cells that secrete T helper 1 (Th1) cytokines (i.e., interleukin-2 [IL-2], interferon-γ [IFN-γ], and tumor necrosis factor-α [TNF-α]), in the systemic circulation and their recruitment into the central nervous system appear to play a crucial role in the pathogenesis of MS.

Chemokines, chemoattractant cytokines that mediate the migration of leukocytes into tissues, and their respective chemokine receptors (CCR) are important molecules for the selective invasion of T cells into target organs. Furthermore, recent data suggest that different subsets of effector T cells can be characterized based on their chemokine receptor profile. Thus, CCR5 has been found to be preferentially expressed on activated Th1 clones. In addition, expression of the cell surface marker CD26, a surface glycoprotein with dipeptidyl peptidase IV activity, has been associated with in vivo activation of T cells.

The purpose of our study was to combine intracellular staining for IL-2, IFN-γ, and TNF-α with immunostaining for CCR5 and CD26 to estimate the frequency of activated Th1 lymphocytes in peripheral blood of MS patients and healthy controls and to detect evidence for an important disease trait.

Patients and Methods

Patients

MS patients (n = 9) had clinically definite or laboratory-assisted definite MS (7 women and 2 men; mean age, 36.5 ± 11 years; mean disease duration, 27.5 months). Eight of the patients had relapsing–remitting MS with an acute exacerbation of the disease, and 1 patient had primary progressive disease with clinical worsening. None of the patients was taking interferon or glatiramer acetate at the time of the study or had received corticosteroids or immunosuppressants in the previous months. Healthy blood donors (n = 17) served as normal age- and sex-matched controls (13 women and 4 men; mean age, 39.3 ± 16 years).

Methods

Peripheral blood was treated for 5 hours with phorbol myristate acetate (10 ng/ml), ionomycin (1 μM; Sigma, Deisenhofen, Germany), and monensin (2.5 μM; Riedel-deHaen, Seelze, Germany), or with monensin (Riedel-deHaen) only. The cells were then washed with Hanks’ balanced salt solution (Sigma) and consecutively fixed in 4% paraformaldehyde solution (Riedel-deHaen) for 10 minutes. After further washing, the cell membrane was permeabilized with Hanks’ balanced salt solution buffer containing 0.1% Saponin (Sigma) and 0.01 M HEPES buffer (Biochrom, Berlin, Germany). The cells were then stained directly with fluorochrome-labeled antibodies (anti-CD3-Cy5, anti-CCR5-phycoerythrin, and anti-CD26-PE, anti-IL-2-fluorescein isothiocyanate [anti-IL-2-FITC], anti-IFN-γ-FITC and anti-TNF-α-FITC; all obtained from Pharmingen, Hamburg, Germany) for 20 minutes at 4°C in the dark. Cells were analyzed on an Epics XL-MCL cytometer (Coulter, Krefeld, Germany). Statistical analysis was performed using SPSS software (SPSS Software GmbH, Munich, Germany). The Mann-Whitney U test was used to compare data between patient and control groups. For analysis of dependent variables within groups, Wilcoxon’s matched pair signed rank test was performed.

Results

Frequency of CCR5+ and CD26+ T Cells

CD3+ lymphocytes were analyzed for the expression of either CCR5 or CD26. The number of CD26+ T cells did not differ significantly between the two groups (71.9% and 72.6%, for controls and patients, respectively; p = 0.81). However, CCR5+ T lymphocytes occurred at a significantly higher frequency in MS patients compared with healthy controls (p = 0.001), as shown in Figure 1.

Cytokine Secretion Profiles of CCR5+ and CD26+ T Lymphocytes after In Vitro Stimulation

In both patients and healthy controls, significant differences in cytokine production of the T-cell subsets were detected after phorbol myristate acetate and ionomycin stimulation; 63.3% of the CCR5+ T cells were...
found to produce IFN-γ compared with 12.5% of CCR5− T cells (p < 0.001 and p = 0.0002, for controls and patients, respectively). Similar results were observed for TNF-α, where 35.9% of CCR5− T cells were positive for staining with TNF-α (p = 0.005 and p = 0.0002). Figure 2 shows a representative FACS analysis from an MS patient with CD3+ cells and the subsequent gating of CCR5+/− subsets and their capacity for IFN-γ production after stimulation for 5 hours.

CD26+ T lymphocytes showed a different cytokine secretion profile. Here, a significantly higher percentage (39.3%) of CD26+ T cells were producers of IL-2 compared with CD26− T cells (19.7%; p < 0.001 and p = 0.0004, for controls and patients, respectively). In contrast, fewer CD26+ T cells expressed TNF-α (p < 0.001 and p = 0.02) and IFN-γ (p < 0.001 and p = 0.004) than the CD26− T lymphocytes.

**Spontaneous Cytokine Secretion of IFN-γ and TNF-α in MS Patients and Healthy Controls**

To examine in vivo activation of the analyzed T-cell subsets, we determined the spontaneous expression of cytokines in unstimulated peripheral blood lymphocytes. We observed a significantly increased number of spontaneously IL-2–expressing CCR5+ T cells in MS patients (2.7 ± 2%) compared with the control group (0.7 ± 0.5%; p = 0.004). Similar results were obtained for IFN-γ (p = 0.03) and TNF-α (p = 0.007).

**Discussion**

Inflammatory disease activity in MS often remains asymptomatic. Therefore, the need for a reliable surrogate marker of disease activity is evident. During the last year, the expression profile of chemokine receptors has been described as a potential marker to identify effector cells in T-cell–mediated diseases.5,7 One finding of our study was an increased number of CCR5+ T lymphocytes in peripheral blood of MS patients who underwent an acute disease exacerbation. By using intracellular cytokine staining, we showed that this T-cell subset secretes high amounts of IFN-γ and TNF-α after in vitro stimulation with phorbol myristate acetate and ionomycin. The principal finding of our study, however, was not only that the total number of CCR5+ T cells was increased in MS patients, but also that a significantly higher proportion of this T-cell population was spontaneously secreting IL-2, IFN-γ,
and TNF-α, which indicates in vivo activation of the cells.

IFN-γ and TNF-α are believed to play an essential role in the immunopathogenesis of MS. The increased production of both cytokines by peripheral blood cells of MS patients has been shown to precede clinical attacks. High levels of TNF-α were detected in cerebrospinal fluid of patients with chronic progressive MS and correlated with the degree of disability. Furthermore, injection of IFN-γ induced exacerbations of the disease.

In our study, combining immunophenotyping with simultaneous intracellular cytokine staining, we demonstrate that the CCR5<sup>+</sup> T-cell subset of peripheral blood lymphocytes is a source of high in vivo secretion of IFN-γ and TNF-α during an acute MS relapse. The pathogenic significance of this CCR5<sup>+</sup> T-cell population in MS is emphasized because their ligands, the chemokines RANTES and MIP-1α, are enriched in cerebrospinal fluid during MS attacks and expressed in demyelinating brain lesions, respectively. In addition, CCR5-expressing lymphocytes are found in actively demyelinating brain lesions of MS patients. It is noteworthy that it has been shown recently that the vast majority of infiltrating cells in synovial fluid of patients with rheumatoid arthritis also express CCR5. Therefore, CCR5 expression appears to mark T cells associated with the capacity to migrate to inflammatory sites in various T<sub>1</sub>-mediated autoimmune diseases.

In conclusion, our findings in a population of MS patients who were selected for high disease activity suggest that CCR5 expression may be a useful marker to identify effector cells in peripheral blood. Longitudinal studies are warranted to determine whether fluctuations in the percentage of CCR5-expressing cells can be used as a tool to monitor disease activity.

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