Analysis of Hypocretin (Orexin) Antibodies in Patients with Narcolepsy

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Study Objectives: We tested the hypothesis that patients with narcolepsy have serum antibodies specific for preprohypocretin and its derivatives.

Design: We tested sera from strictly diagnosed HLA DQB1*0602-positive narcoleptic patients with cataplexy for evidence of autoantibodies against human preprohypocretin, hypocretin 1 and 2, N-terminal leader and C-terminal peptides of preprohypocretin using enzyme-linked immunosorbent assays (ELISA). These results were compared to samples from nonnarcoleptic psychiatric and sleep apnea controls. Laboratory personnel were blinded to subject status.

Setting: Narcoleptic patients and nonnarcoleptic controls were recruited from the Mayo Clinic facilities in Rochester, Minnesota; Scottsdale, Arizona; and Jacksonville, Florida. Laboratory testing was conducted in the Mayo Psychogenomic Laboratory at the Rochester Mayo Clinic.

Participants: A sample of 34 narcoleptic patients and 49 nonnarcoleptic controls.

Interventions: None.

Measurements and Results: ELISA measurements were in optical density. Primary analyses were of the entire narcoleptic and control groups for each potential antigen, and none of the differences reached P values required for significance after Bonferroni adjustment. Secondary analyses by age and sex yielded P values that were significant after Bonferroni adjustment in only 2 cases, but further statistical analyses cast doubt on the veracity of these differences. In all cases where a significant difference was recorded, the hypothesis was not supported because the control optical density reading was higher than the narcoleptic values.

Conclusions: These ELISA assay results do not support the hypothesis that HLA DQB1*0602-positive narcolepsy with cataplexy is associated with serum antibodies against preprohypocretin or its cleavage products.

Key Words: Narcolepsy, antibody, preprohypocretin, hypocretin 1, hypocretin 2.


INTRODUCTION

NARCOLEPSY IS CHARACTERIZED BY SLEEPINESS AND CATAPLEXY. IT IS ATTRIBUTED TO A DEFECT IN THE HYPOCRETIN (OREXIN) NEUROTRANSMISSION system on the basis of animal models and human studies. Dogs with hereditary narcolepsy have been found to have a mutation of the hypocretin receptor-2 gene.1 Transgenic mice with loss of function of the genes encoding preprohypocretin2 and hypocretin receptor-2,3 as well as genetic ablation of orexin neurons,4 also exhibit cataplexy-like behavioral arrests and electroencephalographic activity (eg, sleep-onset rapid eye movement periods) of varying phenotypes. In support of a similar defect in patients with narcolepsy, hypocretin 1 is characteristically undetectable in their cerebrospinal fluid.5,6 No pathogenic mutation has been found in the hypocretin neurotransmission system of patients with narcolepsy, but a mutation in the hypocretin gene (G→T transversion, Leu16Arg) has been described in a single case of early-onset narcolepsy.7 Autopsied brains of patients with narcolepsy have been reported to exhibit loss of hypocretin in the hypothalamic periformal region by radioimmunoassay, by in situ hybridization,7 and by immunohistochemistry.8 Furthermore, a pattern of gliosis described in narcoleptic brain tissue corresponds with the distribution of hypocretin-2 receptor.9 Hypocretin 1 levels in the cerebrospinal fluid also are reduced in patients with paraneoplastic narcolepsy in the context of Ma2-related autoimmune encephalopathy.10 Narcolepsy in humans is rarely familial, and nonfamilial narcolepsy is strongly associated with an HLA-type (DQB1*0602), as reviewed by Chabas et al.11 Collectively, these findings suggest that autoimmunity directed at some element of the hypocretin-secreting pathway may cause narcolepsy.

In this study, we tested the hypothesis that patients with narcolepsy have serum antibodies specific for preprohypocretin and its derivatives.

METHODS

Patient Recruitment and Sampling

This research was reviewed and approved by the Mayo Institutional Review Board, and all subjects gave their informed consent to participate in the study. A sample of narcoleptic patients was recruited from individuals evaluated for possible sleep disorders in the Mayo Sleep Disorders Centers in Rochester, Minnesota; Scottsdale, Arizona; and Jacksonville, Florida. Individuals meeting Mayo Research Criteria for category A narcolepsy were studied.12 In summary, category A subjects have excessive daytime sleepiness, definite cataplexy, mean sleep latency on a Multiple Sleep Latency Test of less than 8 minutes and either 2 sleep-onset rapid eye movement periods on a Multiple Sleep Latency Test or 1 sleep-onset rapid eye movement....
period on the Multiple Sleep Latency Test and a sleep-onset rapid eye movement period on a preceding nocturnal polysomnogram. Those with excessive daytime sleepiness and cataplexy witnessed by a physician with documented recoverable areflexia were also included. Cataplexy severity was not recorded. Only HLA DQB1*0602-positive narcoleptics were eligible. HLA haplotyping was done on genomic DNA harvested from patients’ leukocytes using polymerase chain reaction-sequence specific priming and polymerase chain reaction-sequence specific oligonucleotide probes. Similarly, nonnarcoleptic controls were recruited from the Mayo Sleep Disorders Centers and from other clinical settings. Narcoleptic and control patients were excluded if they had a personal or first-degree family history of medical illness that may affect immune status (eg, immunodeficiency syndrome, HIV infection, or a known autoimmune illness of any kind [eg, rheumatoid arthritis, lupus, type 1 diabetes mellitus, Grave disease, idiopathic Addison disease, multiple endocrinopathy, diabetes insipidus, stiff-person syndrome, multiple sclerosis]), mental retardation, mental illness that may be caused by an autoimmune diathesis (eg, obsessive compulsive disorder or other anxiety disorder), or tic disorder. Patients or controls with a personal or first-degree family history of narcolepsy (eg, onset after brain injury), and patients or controls with a first-degree family history of narcolepsy were also excluded. Sera from 34 narcoleptic patients (average age, 49.8 ± 19.8 years, range 14.5-76.4 years; 2 were <15 years; 6 were 20-30 years of age; 20 females) and 49 controls without known autoimmune diseases (average age 48.9 ± 13.9 years, range 28.4-78.6 years; 23 female, 4 HLA DQB1*0602 positive) were used in this study. Not all samples were tested with each antigen. The number of samples for each test is shown in Table 1. There were no statistically significant differences between the age or the sex distributions of the narcoleptic and control subjects (P = .806 and P = .287 respectively).

### Table 1—ELISA Results

<table>
<thead>
<tr>
<th>Titer</th>
<th>GST-PREPROmN</th>
<th>Cleaved PREPROmN</th>
<th>Hypocretin 1</th>
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<tr>
<td>40</td>
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<td>1.054 ± 0.307</td>
<td>1.983 ± 0.621</td>
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<tr>
<td>80</td>
<td>0.610 ± 0.276</td>
<td>0.762 ± 0.289</td>
<td>1.636 ± 0.587</td>
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<tr>
<td>160</td>
<td>0.414 ± 0.199</td>
<td>0.535 ± 0.246</td>
<td>1.353 ± 0.324</td>
</tr>
<tr>
<td>320</td>
<td>0.268 ± 0.134</td>
<td>0.392 ± 0.213</td>
<td>1.063 ± 0.456</td>
</tr>
<tr>
<td>640</td>
<td>0.178 ± 0.088</td>
<td>0.255 ± 0.148</td>
<td>0.771 ± 0.356</td>
</tr>
<tr>
<td>1280</td>
<td>0.116 ± 0.052</td>
<td>0.174 ± 0.116</td>
<td>0.537 ± 0.262</td>
</tr>
<tr>
<td>2560</td>
<td>0.081 ± 0.032</td>
<td>0.121 ± 0.091</td>
<td>0.353 ± 0.165</td>
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<table>
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<tr>
<th>Hypocretin 2</th>
<th>N-Terminal Peptide</th>
<th>Peptide 1</th>
<th>Peptide 4</th>
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<td>Narcoleptic</td>
<td>Control</td>
</tr>
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<td>n = 20</td>
<td>n = 28</td>
<td>n = 20</td>
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<td>40</td>
<td>0.638 ± 0.382</td>
<td>0.623 ± 0.369</td>
<td>1.398 ± 0.522</td>
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<tr>
<td>80</td>
<td>0.375 ± 0.223</td>
<td>0.344 ± 0.259</td>
<td>0.957 ± 0.402</td>
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<tr>
<td>160</td>
<td>0.295 ± 0.205</td>
<td>0.292 ± 0.254</td>
<td>0.681 ± 0.316</td>
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<tr>
<td>320</td>
<td>0.234 ± 0.177</td>
<td>0.231 ± 0.266</td>
<td>0.479 ± 0.219</td>
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<tr>
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<td>0.179 ± 0.140</td>
<td>0.180 ± 0.205</td>
<td>0.316 ± 0.140</td>
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<tr>
<td>1280</td>
<td>0.141 ± 0.107</td>
<td>0.141 ± 0.154</td>
<td>0.235 ± 0.093</td>
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<tr>
<td>2560</td>
<td>0.125 ± 0.091</td>
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<th>Control</th>
<th>Peptide 3</th>
<th>Narcoleptic</th>
<th>Control</th>
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<tr>
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<td>n = 20</td>
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<td>40</td>
<td>0.304 ± 0.172*</td>
<td>0.434 ± 0.276</td>
<td>0.846 ± 0.497</td>
<td>0.953 ± 0.675</td>
<td>0.390 ± 0.229</td>
<td>0.433 ± 0.281</td>
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<tr>
<td>80</td>
<td>0.181 ± 0.134</td>
<td>0.193 ± 0.172</td>
<td>0.458 ± 0.337</td>
<td>0.554 ± 0.502</td>
<td>0.205 ± 0.107</td>
<td>0.243 ± 0.133</td>
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<tr>
<td>160</td>
<td>0.136 ± 0.095</td>
<td>0.129 ± 0.111</td>
<td>0.321 ± 0.265</td>
<td>0.364 ± 0.351</td>
<td>0.154 ± 0.076</td>
<td>0.184 ± 0.090</td>
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<tr>
<td>320</td>
<td>0.087 ± 0.053</td>
<td>0.094 ± 0.073</td>
<td>0.211 ± 0.175</td>
<td>0.240 ± 0.226</td>
<td>0.118 ± 0.061</td>
<td>0.135 ± 0.057</td>
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</tr>
<tr>
<td>640</td>
<td>0.069 ± 0.032</td>
<td>0.066 ± 0.040</td>
<td>0.129 ± 0.106</td>
<td>0.153 ± 0.135</td>
<td>0.090 ± 0.047</td>
<td>0.111 ± 0.062</td>
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<tr>
<td>1280</td>
<td>0.054 ± 0.024</td>
<td>0.053 ± 0.030</td>
<td>0.083 ± 0.062</td>
<td>0.100 ± 0.075</td>
<td>0.076 ± 0.040</td>
<td>0.094 ± 0.052</td>
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<tr>
<td>2560</td>
<td>0.045 ± 0.021</td>
<td>0.043 ± 0.021</td>
<td>0.059 ± 0.037</td>
<td>0.070 ± 0.041</td>
<td>0.065 ± 0.035*</td>
<td>0.090 ± 0.046</td>
<td></td>
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</tr>
</tbody>
</table>

Results of enzyme-linked immunosorbent assay (ELISA) testing as mean ± SD optical density (OD) of narcoleptic samples compared to control samples. *P ≤ 0.05.

Production of Bacterially Expressed Proteins

Glutathione-S-transferase (GST)-preprohypocretin was generated by inserting the coding cDNA for preprohypocretin into the pGEX-6P vector system using the technique described in the product literature (Amersham, Piscataway, NJ). This system places a GST tag followed by a PreScission™ Protease recognition peptide sequence near the N-terminus of the preprohypocretin polypeptide sequence. An initial attempt to express the entire preprohypocretin sequence was unsuccessful due to the highly hydrophobic characteristics of the leader sequence. Therefore, we inserted cDNA encoding preprohypocretin minus the N-terminal sequence (GST-PREPROmN) following PCR using primers 41++ AAAAAGATCCCTTGCCCCATCTGCTGTCGTGC and 040- AAAAAAATTTCCAGATCCCCAGGTGCTC and obtained good yields in E. coli BL21. A portion of the product was cleaved with
null hypothesis of no difference against 2-sided alternatives.

**Statistical Analysis**

Control versus narcolepsy optical density readings were evaluated for each antigen at each titer using rank sum tests to compare the null hypothesis of no difference against 2-sided alternatives.

The primary analyses focused on the comparisons between all narcoleptic and control sera. Additional comparisons were performed while adjusting for age and sex. Secondary analyses were performed as well. These included separate comparisons for males and females, as well as for those in 2 age groups: those younger than 45 and those at least 45 years of age. Because 9 antigens were assayed at 7 titers each, we accounted for multiple comparisons—separately for primary and secondary analyses—in 2 ways. First, we constructed quantile plots in the spirit of Schweder and Spjotvoll and compared the observed distribution of P values to the uniform distribution that is expected if no tests are truly significant using Kolmogorov-Smirnov tests. These tests provide a single P value for the entire collection of comparisons being considered. In order to discuss P values on an individual basis, we compared the observed P values to Bonferroni corrected levels. We set our level of significance at .05. With 63 tests, the Bonferroni adjusted P value is 7.9 x 10^-4, and with 252 secondary tests of hypothesis, the corrected critical P value is 2.0 x 10^-4.

**RESULTS**

**ELISA Results**

Table 1 shows the results of ELISA testing comparing the entire narcolepsy versus control groups. Optical density readings for assays involving 2 antigens (Peptide 2 and 4) yielded a single P ≤ .05 at 1:40 and 1:2560 dilutions, respectively. In both cases, the average narcoleptic optical density value was less than the average for the control group. Similarly, in the secondary analyses, P ≤ .05 was observed in the male group using peptide 4 at 1:160 and 1:2560 dilutions, in the < 45 years of age group using cleaved PREPROmN (1:1280 dilution) and peptide 1 (1:2580 dilution), and in the ≥ 45 years of age group using cleaved PREPROmN (1:320 dilution), peptide 2 (1:2580 dilution), and GST-PREPROmN (1:40 and 1:80 dilutions). In each case in which P was .05 or less, the average narcoleptic optical density was less than the average control optical density; thus, the results were counter to the hypothesis for this study. In only 2 of these secondary analyses, and none of the primary analyses, did comparisons reach significance after Bonferroni adjustment, with the < 45 years of age group using cleaved PREPROmN at 1:1280 dilution and the ≥ 45 years of age group using peptide 2 at 1:2560 dilution. However, when we compared the observed P-value distribution to what is expected if no tests are significant, the observed deviations were not large enough to reach statistical significance in the primary (P = .1702) or secondary (P = .2796) analyses.

**DISCUSSION**

The strong association between HLA DQB1*0602 and nonfamilial narcolepsy and the association between abnormalities in the hypocretin neurotransmission system and human narcolepsy described in the introduction of this article implicate an autoimmune pathogenic mechanism affecting the hypocretin-secreting cells. Study of the intracellular preprohypocretin polypeptide is relevant because other intracellular antigens serve as markers for autoimmune diseases affecting the central nervous system, such as intracellular glutamic acid decarboxylase-65 kd (GAD65, also known as GAD2) in stiff-person syndrome and paraneoplastic type-1 antineuronal nuclear autoantibody (ANNA-1) in small cell lung carcinoma associated with neuropathies, cerebellar ataxia,
limbic encephalitis, polyradiculopathy, and other neurologic symptoms. In the case of stiff-person syndrome, molecular mimicry is a suspected etiology,\(^\text{17,18}\) and in paraneoplastic small cell lung carcinoma, cancer cell death and lysis are believed to cause exposure of proteins not usually detected by the immune system, which then breaks immune tolerance.\(^\text{19,20}\) We speculate that one or the other mechanism could be operative in narcolepsy. Exposure to an environmental antigen with similarities to an epitope presented by the hypocretin-secreting cells might result in generation of an antibody cross-reactive with and cytotoxic to the hypocretin cells. Alternatively, narcolepsy may not be caused by antibodies against components of the hypocretin neurotransmission system but some other process (eg, a neurodegenerative process or a cell-mediated, nonhumoral, immune process) may cause cell death and release of cellular products in such a way as to break immune tolerance, thus generating an immune marker for the disease.

This is the first study to employ patients’ sera to thoroughly dissect the preprohypocretin polypeptide in an attempt to detect sequences that might be antigenic. We tested 9 candidate antigens derived from preprohypocretin because this molecule is cleaved into several components to produce the neurotransmitters hypocretin 1 and 2. Preprohypocretin and each cleavage product will have a different 3-dimensional structure that would determine the antigenicity of the epitopes to which autoantibodies might bind.

Our results do not support the hypothesis that antibodies directed against preprohypocretin or its peptide components might cause narcolepsy. The differences we found (\(P\geq0.05\)) between narcoleptic and controls were all counter to the hypothesis that narcoleptic patients would have higher antibody titers and, therefore, higher optical density readings. These differences were likely spurious findings because with no antigen did we see significance with all titers, only 2 comparisons in the secondary analysis yielded significance after Bonferroni adjustment, and comparison of observed \(P\)-value distribution using Kolmogorov-Smirnov tests yielded no significance. For biologic significance, one would expect specific antibodies to be abundant in the narcoleptic subjects and to cover a broad range of titers. It is possible, however, that disease-specific antibodies may appear transiently early in the course of narcolepsy and gradually disappear when the antigen-producing cells are lost, as has been reported for pancreatic islet cell antibodies in juvenile diabetes.\(^\text{21}\) It is interesting that, wherever \(P\leq0.05\) was observed, the average control optical density was less than the average control optical density. We cannot rule out the possibility that narcoleptics truly have fewer antibodies than controls and that, with a larger number of subjects, we would more consistently detect significant differences, but it is difficult to explain this finding. We could speculate that narcoleptics, who produce less preprohypocretin in their hypothalamus, may have less autostimulation of their immune system with this antigen, thus producing a lower titer of “background” antibodies. However, we are unaware of any precedent for this in other diseases.

It is of particular interest that there was no evidence for reactivity to the N-terminal region peptide because Siebold et al\(^\text{22}\) showed that the peptide MNLPSKVSVAW, which represents amino acids 1-13 of the N-terminal leader sequence, bound the DQB1*0602 molecule with an apparent affinity that was 10-fold better than that of CSCRLYELLHGG, which is part of the hypocretin-1 sequence. This finding suggested that the N-terminal leader stood a better chance of being the antigen responsible for eliciting autoimmunity in narcolepsy when compared to hypocretin 1. However, we found no evidence for this in our study.

Our study also has technical limitations. The solid phase binding of antigen in our assays limits the 3-dimensional characteristics of the polypeptide antigens to the point that they might no longer bind to antibodies in patient sera, which could produce a false-negative result. However, our use of the GST-PREPROmN antigen bridged to the plate by glutathione would circumvent this problem to a considerable extent. Binding of the antigen by the GST tag would allow the antigenic molecule to be in solution phase to assume a more native conformation. Another limitation was the fact that we could not test a full-length preprohypocretin polypeptide fusion protein because of its low yields from the expression system used for its production. However, we did test a synthetic peptide corresponding to the leader sequence separately as an alternative form of this candidate antigen to compensate for this problem and found no reactivity.

This work compliments previous work in which we looked for neuronal-specific antibodies using neuroimmunologic methods\(^\text{23}\) and where immunoprecipitation assays, western blot analysis, and immunofluorescence microscopy was done using serum and cerebrospinal fluid samples of HLA DQB1*0602 patients yielding no evidence of autoimmunity (unpublished data). This work also is consistent with the findings by Taheri et al,\(^\text{24}\) in which western blot analysis of protein from the lateral hypothalamus of dogs, rats, and mice using sera and cerebrospinal fluid from HLA DQB1*0602 and hypocretin-deficient narcoleptics, yielded negative results and is consistent with the work by Chabas et al\(^\text{25}\) who reported that no antibodies against hypocretin 1 and 2 were detected on ELISA. However, these collective works do not nullify the hypothesis that autoimmunity causes narcolepsy because only a few candidate antigens have been tested to date. The hypothesized autoimmune response directed against the hypocretin-secreting cells may be directed at another antigen on the cell. Specifically, the hypocretin-secreting cells have a number of cell-surface antigens that make attractive candidates for future studies. A partial list of antigens includes hypocretin receptors 1 and 2 and insulin, leptin, norepinephrine, serotonin, and glutamate receptors.\(^\text{25}\) Similarly, an autoimmune mechanism might be affected by cytotoxic T lymphocytes rather than an autoantibody-mediated mechanism. Finally, it is conceivable that antibodies may be restricted to the cerebrospinal fluid, as has been reported in some patients with paraneoplastic autoimmunity.\(^\text{26}\)

In summary, we found no evidence for immunoreactivity on ELISA using GST-PREPROmN, cleaved PREPROmN, hypocretin 1, hypocretin 2, N-terminal peptide, and 4 overlapping peptides encoding the carboxyl-terminal region of preprohypocretin when we tested sera of patients with HLA DQB1*0602 narcolepsy with cataplexy and compared to controls.

ACKNOWLEDGEMENTS

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REFERENCES


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