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Anti-U1 RNP antibodies in cerebrospinal fluid are associated with central neuropsychiatric manifestations in systemic lupus erythematosus and mixed connective tissue disease

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Objective. To determine the significance of anti-U1 ribonucleoprotein (RNP) antibodies (Abs) in cerebrospinal fluid (CSF) from systemic lupus erythematosus (SLE) and mixed connective tissue disease (MCTD) patients with central neuropsychiatric (NP) SLE.

Methods. Antinuclear Abs including anti-U1RNP Abs were determined in sera and CSF of 24 patients with SLE and four patients with MCTD and central NPSLE using an RNA immunoprecipitation assay and ELISA. The frequency of CSF anti-U1RNP Abs in patients with central NPSLE was examined, and the anti-U1RNP index (= [CSF anti-U1RNP Abs /serum anti-U1RNP Abs] / [CSF IgG /serum IgG]) was compared with CSF interleukin (IL)-6 levels and the albumin quotient (*Q*alb, an indicator of blood brain barrier damage). CSF and serum Abs against U1-70K, A, and C including autoantigenic regions were examined, and the anti-70K, A, and C indices as well as the anti-U1RNP index were calculated.

Results. CSF anti-U1RNP Abs with an increased anti-U1RNP index showed 64% sensitivity and 93% specificity for central NPSLE. The anti-U1RNP index did not correlate with CSF IL-6 levels or *Q*alb. The anti-70K index was higher than the anti-A and C indices in CSF of anti-U1RNP Ab-positive patients with central NPSLE. The major autoantigenic

region for CSF anti-70K Abs appeared to be localized in the U1-70K 141–164 amino acid residues within the RNA-binding domain.

Conclusions. CSF anti-U1RNP Abs and the anti-U1RNP index are useful indicators for central NPSLE in anti-U1RNP Ab-positive patients. The predominance of anti-70K Abs in CSF suggests intrathecal anti-U1RNP Ab production.

Systemic lupus erythematosus (SLE) is a chronic, remitting and relapsing, multisystem autoimmune disease that predominantly affects women. Among various organ damages, neuropsychiatric SLE involving the central nervous system (CNS; central NPSLE) is a life-threatening severe manifestation. The overall prevalence of NPSLE varies between 14 and 75% in a representative selection of studies using the American College of Rheumatology (ACR) nomenclature (1). In fact, NPSLE may affect mortality in SLE and is associated with a significant negative impact on a patients' quality of life. However, 41% of all CNS manifestations in SLE patients may be attributed to factors other than lupus (2).

Although the pathophysiology of most NPSLE cases is not well determined, a particular subset of autoantibodies (Abs) is associated with neuronal injury. A subset of anti-DNA Abs cross-reacts with a sequence within the *N*-methyl-D-aspartate receptor subunit NR2 (anti-NR2 Abs), causing excitatory synaptic transmission in the CNS (3). Anti-NR2 Abs preferentially target the hippocampus, suggesting that anti-NR2 Abs may disrupt normal cognitive processes (4). However, serum anti-NR2 Abs may be associated with depressive mood (5), but not cognitive dysfunction (5, 6). Arinuma et al. (7) reported that anti-NR2 Abs in cerebrospinal fluid (CSF), but not in serum, were involved in diffuse central NPSLE.

Anti-ribosomal P Abs have been reported as neurotoxic autoAbs (8–10). Abnormal behavior can be induced in normal mice by administering anti-ribosomal P Abs (10), although serum anti-ribosomal P Abs are not specific to human central NPSLE (11). In association with thrombosis, anti-β2-glycoprotein I and prothrombin Abs are recognized as pathogenic Abs in focal central NPSLE such as cerebrovascular disease (12).

In addition to autoAbs, several proinflammatory cytokines and chemokines have been detected in serum and CSF of patients with SLE. The most important cytokine in SLE and NPSLE may be interferon (IFN)-α (13–15). A recent report showed the presence of IFN-α, IFN-γ-inducible protein 10 (IP-10), interleukin (IL)-8, and monocyte chemotactic protein (MCP)-1 in the CSF from patients with NPSLE (15). IFN-α generation in SLE is caused, at least partially, by autoAbs that bind to ribonucleoprotein (RNP) particles released from dead and dying cells. Santer et al. clearly suggested that IFN-inducing activity in CSF correlates with serum anti-U1RNP Abs, but not with other known antinuclear antibodies (ANA) (15). Therefore, anti-U1RNP Abs and their immune complex in CSF may have pathogenic roles in central NPSLE. Okada et al. reported 14 patients with aseptic meningitis (eight with SLE, six with mixed connective tissue disease [MCTD] or undifferentiated

connective tissue disease, and one with Sjögren's syndrome) among 1560 patients with connective tissue disease. Serum anti-U1RNP Abs were positive in 13 of the 14 patients with aseptic meningitis, suggesting that anti-U1RNP Abs may be linked to central NP manifestations (16). In the present study, we evaluated the clinical significance and immunological characteristics of anti-U1RNP Abs in CSF derived from SLE and MCTD patients with central NP manifestations.

PATIENTS AND METHODS

Patients. Patients with SLE or MCTD who revealed CNS manifestations and had been admitted to the Department of Rheumatology and Clinical Immunology, Kyoto University Hospital, from March 2002 to October 2007 were enrolled. SLE was diagnosed according to the ACR criteria (17, 18) and MCTD was diagnosed according to the criteria proposed by the Ministry of Health and Welfare in Japan (19). CNS manifestations were classified according to the case definitions for NP syndromes in SLE (20). Evaluation of NP syndromes included neuropsychiatric testing and magnetic resonance imaging (MRI) of the brain. Patients were diagnosed with central NPSLE retrospectively, according to

Kwiecinski's criteria (21) with some modifications. Briefly, central NPSLE was defined as the presence of at least two of the following six items: 1) recent-onset of psychosis, 2) transverse myelitis, 3) aseptic meningitis, 4) seizures, 5) pathologic changes visualized on brain MRI, and 6) severely abnormal cognitive dysfunction documented by neuropsychiatric testing. Oligoclonal IgG bands in the CSF were not included, and CNS manifestations caused by other factors (e.g., concurrent non-SLE NP disease such as infection and secondary NPSLE such as uremia, hypertension, and complications of SLE therapy) were not defined as central NPSLE. In the present study, small punctate focal lesions in white matter, cortical atrophy, periventricular white matter hyperintensity, diffuse white matter changes, discrete gray matter lesions, diffuse gray matter hyperintensities, cerebral edema, and new infarct were considered as pathologic changes on brain MRI. The present study was conducted in compliance with the Declaration of Helsinki and approved by the Kyoto University Ethics Committee Review Board (approval #E97).

Samples. After an acute phase of massive cerebrovascular disease (CVD) was ruled out by brain MRI screening, CSF was taken from patients within 3 days from the onset of CNS manifestations. We have a written informed consent from all of the studied patients.

Serum samples were collected from all patients on the same day, and both samples were stored at -80°C. Routine CSF and serum analyses, including total protein, albumin, and IgG level, were conducted. CSF interleukin (IL)-6 (R&D Systems, Minneapolis, MN, USA) and interferon (IFN)-α (Bender MedSystems, Vienna, Austria) levels were determined by ELISA, according to the manufacturer's protocol. The sensitivity of the IFN-α assay was more than 5 pg/mL.

***Q*alb and IgG index.** To determine blood brain barrier (BBB) damage, the albumin quotient (*Q*alb, normal < 0.0076) was calculated as the ratio of CSF albumin (mg/dL) to serum albumin (mg/dL) (22). The IgG index was calculated as the IgG ratio (CSF IgG concentration [mg/dL] /serum IgG concentration [mg/dL])/*Q*alb.

Detection of antinuclear antibodies (ANA) in sera and CSF by RNA immunoprecipitation. RNA immunoprecipitation (RNA-IPP) using HeLa cell extracts was performed to determine anti-RNA-associated antigen Abs, such as anti-U1RNP, Sm, SS-A/Ro (SSA), SS-B/La (SSB), and ribosomal P Abs in sera and CSF (23). A 10 µl sample of sera or CSF was mixed with 2 mg of protein A Sepharose™ CL-4B (GE Healthcare, Upsala, Sweden) in 500 µl of IPP buffer (10 mM Tris-HCl at pH 8.0, 500 mM NaCl, 0.1%

Nonidet P-40 [NP-40]) and incubated on a rotator for 2 h at 4°C. The IgG-coated Sepharose was washed four times in 500 µl of IPP buffer using 10-s spins in a microfuge and resuspended in 400 µl of NET-2 buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.05% NP-40). For RNA analysis, this suspension was incubated with 300 µl of HeLa cell extracts, derived from 6×10^6 cells, on a rotator for 2 h at 4°C. The antigen-bound Sepharose was then collected by a 10-s centrifugation in a microfuge, washed four times with 500 µl of NET-2 buffer, and resuspended in 270 ml of NET-2 buffer. To extract bound RNAs, 30 µl of 3.0 M sodium acetate, 15 µl of 20% sodium dodecyl sulfate, and 300 µl of phenol/chloroform/isoamyl alcohol (50:50:1; containing 0.2 g of 8-hydroxyquinoline and 40 ml of 0.1 M Tris-HCl at pH 7.5) were added to the Sepharose beads. After agitation in a vortex mixer and spinning for 1 min, RNAs were recovered in the aqueous phase after ethanol precipitation and dissolved in 20 µl of electrophoresis sample buffer composed of 10 M urea, 0.025% bromphenol blue, and 0.02 % xylene cyanol FF (Bio-Rad, Hercules, CA, USA) in Tris-borate-EDTA buffer (90 mM Tris HCl at pH 8.6, 90 mM boric acid, and 1 mM EDTA). The RNA samples were denatured at 65°C for 5 min and then resolved by 7 M urea-10% polyacrylamide gel electrophoresis with silver staining (Bio-Rad). Anti-U1RNP,

Sm, SSA, and SSB Abs were determined as positive when U1RNA, U1-U6RNA, SSA-RNA (Y1-Y5RNA), and SSB-RNA (5S-ribosomal RNA, 7S-RNA, and Y1-Y5RNA) were precipitated, respectively.

Detection of ANA in sera and CSF by ELISA. Serum anti-U1RNP, dsDNA, SSA, and ribosomal P Abs were also determined by ELISA using recombinant U1RNP (Mesacup®-2 test RNP, Medical and Biological Laboratories [MBL] Co., Nagoya, Japan), purified dsDNA (Mesacup DNA-II test ds®, MBL Co.), recombinant SS-A (Mesacup®-2 test SS-A, MBL Co.), and recombinant ribosomal P proteins (Ribosomal P ELISA kit, MBL Co.), according to the manufacturer's protocol. Anti-ribosomal P and dsDNA Abs in CSF were determined to be positive when the experimental titer was more than that of the mean + 2SD of 10 negative controls. CSF samples derived from patients with other autoimmuine diseases (4 with polyarteritis nodosa, 4 with multiple sclerosis, and 2 with rheumatoid arthritis) were used as negative CSF controls for the anti-ribosomal P and dsDNA Abs. These patients had CNS manifestation and/or CSF abnormality, but not NPSLE, and negative results for their serum ANA were confirmed. Sera were diluted 1:100, and CSF samples were diluted 1:5 using phosphate buffer saline (PBS). To determine the levels of anti-70K, A, and C Abs,

recombinant U1-70K, A, and C (MBL Co.) along with coating buffer (pH 9.4) (1 μg/mL) were bound to ELISA plates, and the wells were blocked using 5% bovine serum albumin (BSA) in PBS. Patient sera and CSF containing anti-U1RNP Abs were added and incubated at room temperature for 2 h, followed by detection of bound IgG with alkaline phosphatase-conjugated anti-human IgG (Southern Biotechnology Associates Inc., Birmingham, AL, USA) at $OD_{405\ nm}$ in a microtiter ELISA reader. All assays were performed in triplicate. We confirmed that the OD_{405nm} values of the CSF-experimental wells were included within the linear range of the positive control. If a 1:5 dilution was not appropriate, the dilution was changed in such samples and the obtained titer was adjusted.

Anti-U1RNP ratio and anti-U1RNP index. Arbitrary units of the anti-U1RNP Abs in each sample were determined using serum and CSF anti-U1RNP Ab-positive (defined as 100 units) and negative standard samples. More precisely, the arbitrary units for serum or CSF anti-U1RNP Abs were calculated as: ([$OD_{405\ nm}$ of experimental well − $OD_{405\ nm}$ of anti-U1RNP Ab-negative standard well] × 100/[$OD_{405\ nm}$ of anti-U1RNP Ab-positive standard well − $OD_{405\ nm}$ of anti-U1RNP Ab-negative standard well]). After obtaining the arbitrary units of the anti-U1RNP Ab, the anti-U1RNP ratio and anti-U1RNP index were

calculated as: (CSF arbitrary anti-U1RNP Ab units × 5/serum arbitrary anti-U1RNP Ab units × 100) and anti-U1RNP ratio/IgG ratio, respectively. The difference in the dilutions between serum (×100) and CSF (×5) was adjusted, and the anti-70K, A, and C indices were obtained by the same calculation method.

Determination of autoantigenic regions recognized by serum and CSF anti-70K Abs. To compare the autoantigenic regions in the U1-70K proteins recognized by the CSF and serum anti-U1RNP Abs, we prepared 22 overlapping synthetic peptides (17–24 amino acids [aa]) identical with the U1-70K partial sequences (24) (Fig. 4). Synthetic peptides (100 μg/ml) with coating buffer (pH 9.4) were bound to ELISA plates and the wells were blocked with 5% BSA in PBS. Sera and CSF from eight patients with central NPSLE were diluted to 100 μg/mL with PBS and then incubated for 2 h at room temperature, followed by detection of bound IgG with alkaline phosphatase-conjugated anti-human IgG (Southern Biotechnology Associates Inc.) at $OD_{405\ nm}$ in a microtiter ELISA reader. All assays were performed in triplicate. Positivity was determined as more than the mean + 2SD of the negative control samples obtained from patients without serum and CSF anti-U1RNP Abs.

Statistical analysis. Differences in the frequencies of ANA in CSF and serum were

evaluated by the chi-square (χ^2) test and Fisher's exact test as appropriate. The Student's *t*-test was used to compare the difference between two group means. Pearson's product moment correlation coefficients (*r*) were calculated to evaluate the correlation between the anti-U1RNP Ab index and other indicators. A *p* value less than 0.05 was considered significant.

RESULTS

Patient characteristics. NP syndromes were detected in 24 patients with SLE and four patients with MCTD. All patients were female, and their age at onset of CNS manifestations was 34.1 years (range, 19–58 years). According to the ACR nomenclature (20), the symptoms of central NPSLE exhibited by our patients were as follows: headache, cerebrovascular disease, cognitive dysfunction, seizures and seizure disorder, aseptic meningitis, psychosis, acute confusional state, demyelinating syndrome, anxiety disorder, and movement disorder.

CSF findings and ANA profiles in patients with central NPSLE. Fourteen patients were diagnosed with central NPSLE (Table 1). All patients except for patient 11 needed a

high-dose corticosteroid treatment along with methylprednisolone pulse therapy and/or immunosuppressive agents for their central NPSLE. Aseptic meningitis most frequently occurred (five patients). Of the 14 patients, the cell number in CSF increased in six patients (46%, normal < 4 cells/μl), and total protein concentration increased in eight patients (57%, normal < 40 mg/dL). CSF IL-6 was elevated in five of 12 patients (42%, normal < 4.3 pg/mL [25]), and the IgG index increased in nine of 14 (64%, normal < 0.67). *Q*alb, an index of blood brain barrier (BBB) permeability, increased in eight of 14 patients (57%, normal < 0.0076 [22]). By RNA-IPP, anti-U1RNP, SSA, SSB, and Sm Abs were detected in sera from 11 (79%), eight (57%), one (7%), and four (29%) patients, respectively. Anti-ribosomal P and dsDNA Abs were determined as positive by ELISA in sera from four (29%) and six (43%) patients, respectively. In contrast, anti-U1RNP Abs in CSF were most frequently detected by RNA-IPP (82%) in CSF from anti-U1RNP Ab-positive patients with central NPSLE (Fig. 1 and 2A). The anti-U1RNP ratios increased more than the IgG ratios in patients with CSF-anti-U1RNP Abs (Fig. 2B). CSF anti-SSA and dsDNA Abs were detected in only three and one patient, respectively. Anti-ribosomal P and Sm Abs were absent in CSF.

CSF findings and ANA profiles in patients without central NPSLE. CNS

manifestations were diagnosed in 14 patients as concurrent non-SLE central NP diseases or secondary central NP syndromes (Table 2). Increased cell number and protein concentrations were observed in three (21%) and five (36%) patients, respectively. IL-6, the IgG index, and *Q*alb were elevated in four (40%), seven (54%), and two (15%) out of 14 patients, respectively. These values were not significantly different between patients with and without central NPSLE. In contrast to central NPSLE, CSF anti-U1RNP Abs were detected in only one patient (#15) without central NPSLE (Fig. 2A), whereas serum anti-U1RNP Abs were detected frequently (71%).

Correlation of anti-U1RNP index to CSF IL-6 level and *Q*alb. The anti-U1RNP index was compared to CSF IL-6 and *Q*alb determined from the same samples in 10 CSF anti-U1RNP Ab-positive patients (# 1–9, and 15). The anti-U1RNP index was independent of the CSF IL-6 (Fig. 2C) and anti-U1RNP ratio was not correlated to the IgG indices (Fig. 2D). *Q*alb, which is reportedly a useful BBB permeability indicator, tended to inversely correlate with the anti-U1RNP index (Fig. 2C, $y = -0.246x + 9.37$).

Anti-70K Abs were most dominant in CSF among the Abs against U1RNP-specific proteins. Anti-U1RNP Abs usually recognize either U1-70K, A, or C

proteins, which are the unique components of the U1RNP particle. We examined the anti-70K, A, and C indices as well as the anti-U1RNP index in eight CSF anti-U1RNP Ab-positive patients with central NPSLE (refer to *Table 1*), and the anti-U1RNP index was greater than 2.0 in all patients. In the same samples, there was an elevation of the anti-70K index (> 1.0) in seven of eight patients, whereas the anti-A and C indices were elevated in only a few patients. The average anti-70K index was significantly higher than that of the anti-C index and tended to be higher than that of the anti-A index (Fig. 3).

U1-70K autoantigenic regions recognized by serum and CSF anti-U1RNP Abs. More detailed reactivity of the U1-70K protein was examined in sera and CSF from eight patients with CSF anti-U1RNP Abs. All prepared synthetic peptides were recognized by the eight anti-U1RNP Ab-positive sera, whereas there were 4–21 reacted residues for each serum sample (data not shown). Serum anti-U1RNP Abs in patients with central NPSLE bound the 61–84 aa residue most frequently (88%) (Fig. 4). In contrast, the synthetic peptides that bound CSF anti-U1RNP Abs were limited to 20 residues, and 1–12 was the reacted residue number for each CSF sample (data not shown). The 141–164 aa residue alone was recognized by the majority of the CSF anti-U1RNP Abs (88%), whereas autoepitope pattern

recognized by serum- and CSF-anti-U1RNP Abs was not significantly different (Fig. 4).

DISCUSSION

We showed that CSF anti-U1RNP Abs determined by RNA-IPP and/or an elevation in the anti-U1RNP index are more specific markers for central NPSLE than the CSF IL-6 and IgG indices in serum anti-U1RNP Ab-positive SLE and MCTD patients. This is the first report showing the clinical significance of CSF anti-U1RNP Abs. While other autoAbs such as anti-ribosomal P, NR2, or dsDNA Abs in association with NPSLE have been described, there is no report regarding serum or CSF anti-U1RNP Abs in an international cohort or large study (26, 27). Recently, the sensitivity and specificity of CSF IL-6 (>4.3 pg/mL) for diagnosing central NPSLE have been reported as 87.5% and 92.3%, respectively (25). However, CSF IL-6 is not specific for disease-associated NPSLE because an IL-6 elevation can also be caused by infectious meningoencephalitis and cerebrovascular disease. The IgG index is also elevated in patients without CNS involvement (28), and no statistical differences in IL-6 and the IgG index were observed between central NPSLE and other CNS manifestations in the present study. *Q*alb elevation is strong evidence for BBB damage (22),

and more than half of our patients with central NPSLE had increased BBB permeability, similar to that reported in a previous study (29). However, the BBB damage is caused not only by central NPSLE but also by other factors. *Q*alb was elevated in patients with drug-induced aseptic meningitis (# 21) and miliary tuberculosis (# 22). The presence of anti-U1RNP Abs in CSF along with an anti-U1RNP index of more than 2.0 was frequently observed in patients with, but not without, central NPSLE (sensitivity = 64.3%, specificity = 92.9%). Moreover, the sensitivity and specificity of CSF anti-U1RNP Abs for central NPSLE in serum anti-U1RNP Ab-positive patients were calculated as 81.8% and 90.0%, respectively, While global penetration of serum Abs into CSF occurs from a serious BBB injury, elevation of the anti-U1RNP index is not influenced, because the IgG ratio increases simultaneously in this condition. Thus, the increased anti-U1RNP Ab titer is a possible diagnostic marker for CNS manifestations attributable to SLE or MCTD independent of CSF IL-6 level, IgG index, or *Q*alb.

In a previous study, a correlation was observed between serum anti-Sm Abs and central NPSLE (30), whereas the present study demonstrated no association between the presence of serum anti-U1RNP/Sm Abs and central NPSLE. To date, only our group (31)

and a German group (32) have published case reports of CSF anti-U1RNP Ab-positive patients. The important point of our study may be the use of RNA-IPP for detecting anti-U1RNP Abs. RNA-IPP is the most sensitive and specific method among the immunological methods used for detecting Abs, especially against RNA or RNA-binding proteins (33). The absence of anti-Sm Abs may not be due to a sensitivity problem with the RNA-IPP, because we could not detect the Abs in the same samples by ELISA. Because of the lower amount of cellular SSA-RNAs than U1RNAs, SSA-RNAs are more difficult to visualize than U1RNAs by RNA-IPP. It is possible that anti-U1RNP Abs were unequivocally detected in this system, and the present study may not indicate that an intrathecal ANA stimulation is specific to anti-U1RNP Abs.

The striking deficit in CNS pathology, specifically the lack of vasculitis or massive cellular infiltrate in patients dying of central NPSLE, suggests that the pathogenesis differs from immune complex (IC) deposition, which is a characteristic of lupus nephritis. Rather, anti-U1RNP Abs may act as an inducer of proinflammatory cytokines. It is worth noting that serum and CSF of patients with NPSLE show abnormally high IFN-α-inducing activity (15). In addition to IFN-α and IC formed by CSF, autoAbs produce significantly increased levels

of IFN-γ-inducible protein 10 (IP-10/CXCL), IL-8, and monocyte chemotactic protein-1 (34-36), and this phenomenon is most distinguished in the serum of anti-U1RNP Ab-positive patients (15). It is interesting that this hypothesis was demonstrated, because IFN-α is strongly induced by U1RNP-containing IC (37) and is the key cytokine for SLE pathogenesis. IC and IFN-α were not detected in most of our CSF samples (data not shown), possibly because CSF IFN-α was disrupted quickly and IC moved to FcγR-expressing cells.

Thus far, it has been reported that a certain ANA subset is relevant to central NPSLE. First, serum anti-ribosomal P Abs are definitely a useful diagnostic marker for SLE (38); however, it is controversial whether anti-ribosomal P Abs in serum and/or CSF are a link to central NPSLE or not (11, 38), whereas the presence of ribosomal P protein on the endothelial cells has been demonstrated (39). It is likely that anti-ribosomal P Abs might not be able to pass effectively through the BBB due to binding to CNS endothelial cells (8). When paired serum and CSF samples were diluted to the same IgG concentrations and used for Western blotting, selective enrichment of IgG anti-ribosomal P occurred in the CSF of a few patients (39). Anti-ribosomal P Abs were not detected in CSF from our patients. Second, Kowal et al. have reported that a certain anti-dsDNA Ab subset in CSF, which cross-reacts

with the NR2 glutamate receptor, causes apoptotic neuronal death in the mouse hippocampus (3). Although the CSF level of anti-NR2 Abs was higher in patients with central NPSLE than that in other SLE groups, the highest CSF anti-NR2 Ab levels have been detected in patients with septic meningitis (26), suggesting that anti-NR2 Abs attack neurotransmitters directly through a breach in BBB integrity. While serum and CSF anti-NR2 Abs were not investigated in the present study, the anti-NR2 Ab-mediated neuronal diseases are different than aseptic meningitis and appear to be associated with anti-U1RNP Abs. Unfortunately, the present study did not clearly indicate which types of central NPSLE that anti-U1RNP Abs are most associated with. In accordance with a previous study (27), our data show no significant correlation between the presence of serum- or CSF-anti-SSA Abs and central NPSLE.

The presence of ANA in CSF can be explained by three mechanisms: (1) in situ Ab production in the CNS, (2) a BBB breach, which would allow Abs to cross a normally restricted compartment, and (3) an increased Ab concentration resulting from a reduced CSF flow rate. However, our data strongly suggest intrathecal production of anti-U1 RNP Abs, because the anti-70K, A, and C indices were not equally elevated, and the anti-U1RNP and

70K indices in most patients increased by more than 2.0. Even if a BBB breach or reduced flow occurred, it is unlikely that either anti-70K, A, or C Abs penetrated or moved outside the CSF. The observation that a different U1-70K peptide recognition pattern by serum and CSF anti-70K Abs is evident also suggests intrathecal anti-U1RNP Ab production. Remarkably, the autoantigenic 141–164 aa residue for CSF anti-70K Abs in patients with central NPSLE was located within the RNA-binding domain (92–202 aa), including T (40) and B (41–44) cell major epitopes of human anti-70K Abs. Guldner et al. identified the 56–195 aa domain as the major antigenic epitope recognized by all tested sera (41). Cram et al. reported the 100–156 aa residue as one of the major epitopes in the human 70K protein (42). James et al. showed that the basic aa-rich sequences are the early autoantigenic determinants of the 70K C-terminus (45). More detailed experiments using a large number of CSF samples are necessary to clarify the immunological characteristics of intrathecal anti-U1RNP Abs, whereas our results suggest the possibility that an ANA production in CSF is stimulated by an antigen-driven mechanism.

In conclusion, CSF anti-U1RNP Abs, which may be produced in the CNS, are a clinically useful indicator for central NPSLE. However, there are some limitations to the

present study. First, the usefulness of the anti-U1RNP index is limited to patients with NPSLE that have serum anti-U1RNP Abs. Second, RNA-IPP method may be required to determine a low anti-U1RNP Ab titer, and third, our results were observed in a small number of Japanese patients. A more detailed association of CSF anti-U1RNP Abs with other humoral factors or activated neuronal cells in the CNS should be elucidated in a future study.

AUTHOR CONTRIBUTIONS

Dr. Fujii had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design.	Fujii, Mimori
Patient recruitment.	Imura, Yukawa, Kawabata, Nojima, Ohmura, Usui
Acquisition of data.	Sato, Yokoyama
Manuscript preparation.	Sato, Fujii
Statistical analysis.	Sato, Fujita
Interpretation of data.	Mimori

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FIGURES

Figure 1.

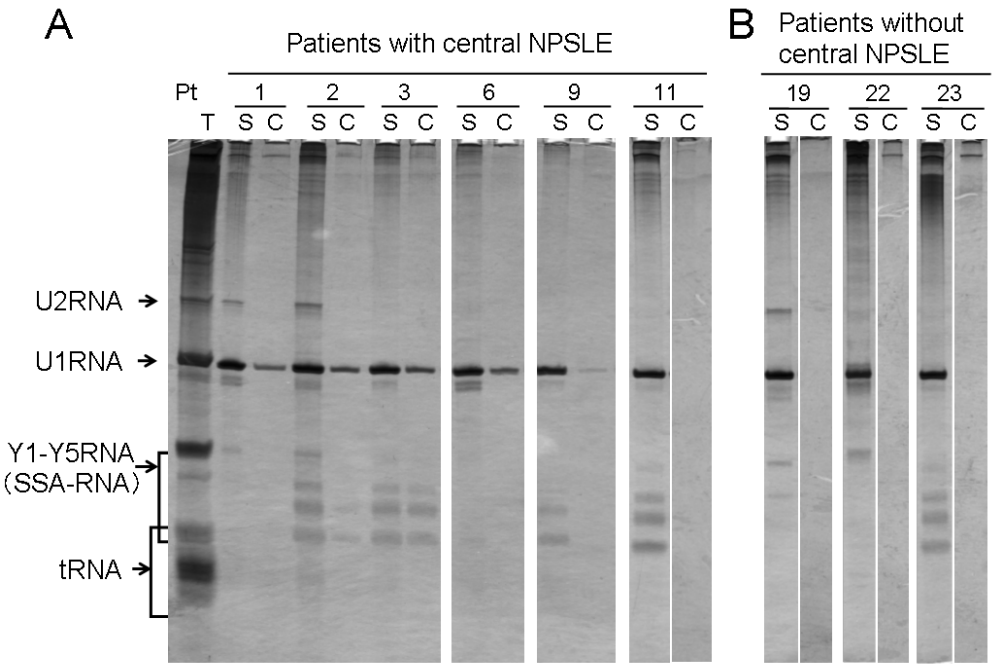

Figure 1. Antinuclear antibody (ANA) detection in sera and CSF by the RNA immunoprecipitation (RNA-IPP) assay. Anti-U1RNP and anti-SS-A/Ro (SSA) Abs precipitated U1RNA and SSA-RNA, respectively. CNS manifestations are listed in Table 1 and 2. (**A**) Serum and CSF samples from representative patients with central neuropsychiatric systemic lupus erythematosus (NPSLE). (**B**) Serum and CSF samples from representative patients without central NPSLE.

Figure 2.

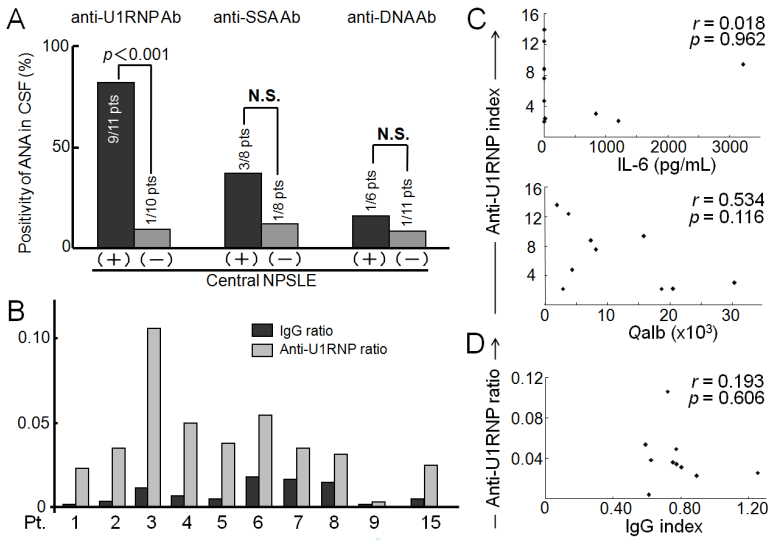

Figure 2. (A) ANA frequency difference in CSF from patients with and without central neuropsychiatric systemic lupus erythematosus (NPSLE). The frequency (%) of CSF ANA-positive in serum ANA-positive patients was shown. Anti-U1RNP and SS-A/Ro Abs were determined by RNA-IPP, and anti-dsDNA Abs were determined by ELISA. Anti-SSB/La and ribosomal P Abs were not detected in CSF. ANA, antinuclear antibodies; CSF, cerebrospinal fluid; IL-6, interleukin-6; Pts, patients; SSA, SS-A/Ro; N.S., not significant. **(B)** Comparison of the IgG and anti-U1RNP ratios. **(C)** Correlations between the anti-U1RNP index and IL-6, or *Q*alb. **(D)** Correlation between the anti-U1RNP ratio and the IgG index. The Pearson's product moment correlation coefficients (r) were calculated in 10 patients with positive CSF anti-U1RNP Abs. IgG ratio = (CSF IgG concentration [mg/dL]/serum IgG concentration [mg/dL]), Anti-U1RNP ratio (with a compensation for the difference in serum/CSF dilutions) = (100/5) × (CSF anti-U1RNP Ab arbitrary units/serum anti-U1RNP Ab arbitrary units), *Q*alb (Albumin quotient, normal < 0.0076) = albumin ratio = (CSF albumin concentration [mg/dL]/serum albumin concentration [mg/dL]), IgG index = IgG ratio (CSF IgG concentration [mg/dL]/serum IgG concentration [mg/dL])/*Q*alb, Anti-U1RNP index = (anti-U1RNP ratio/IgG ratio)

Figure 3.

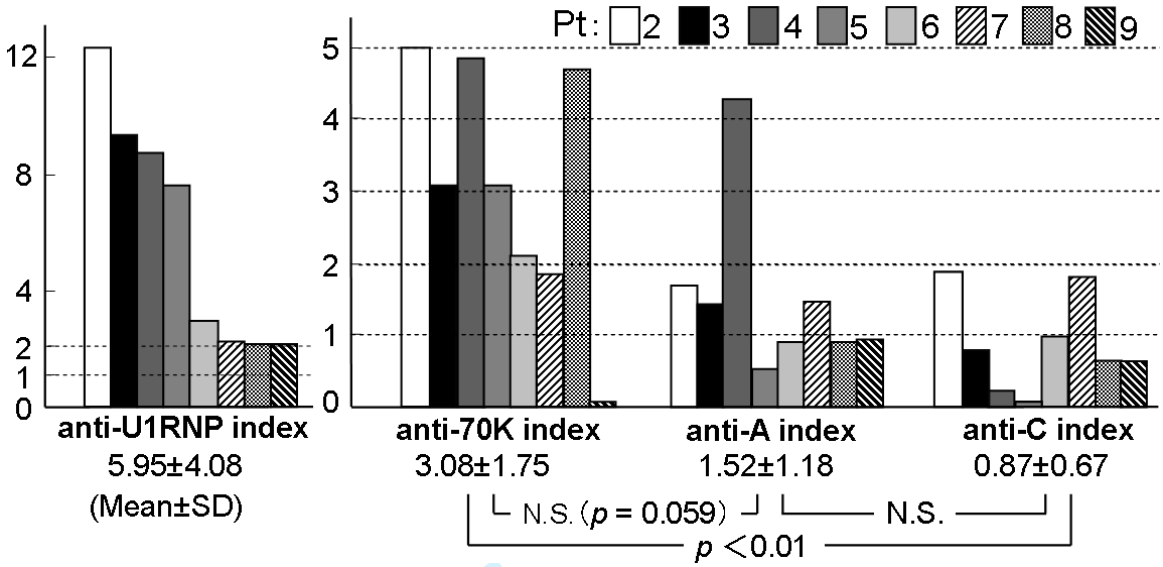

Figure 3. Anti-U1RNP index in eight patients with central neuropsychiatric systemic lupus erythematosus (left) and the anti-70K, A, and C indices in the same patients (right) are shown. Serum and cerebrospinal fluid anti-70K, A, and C Ab titers were determined by ELISA using recombinant proteins. The patient number corresponds to the Pt number in Table 1. Because an insufficient quantity of sample was obtained from Pt.1, the Pt.1 index was excluded from this experiment.
Anti-U1RNP, 70K, A, and C indices (with a compensation for the serum/CSF dilution difference) = (100/5) × ([CSF Ab arbitrary units/serum Ab arbitrary units]/[IgG ratio])
Pt, patient; N.S., not significant

Figure 4.

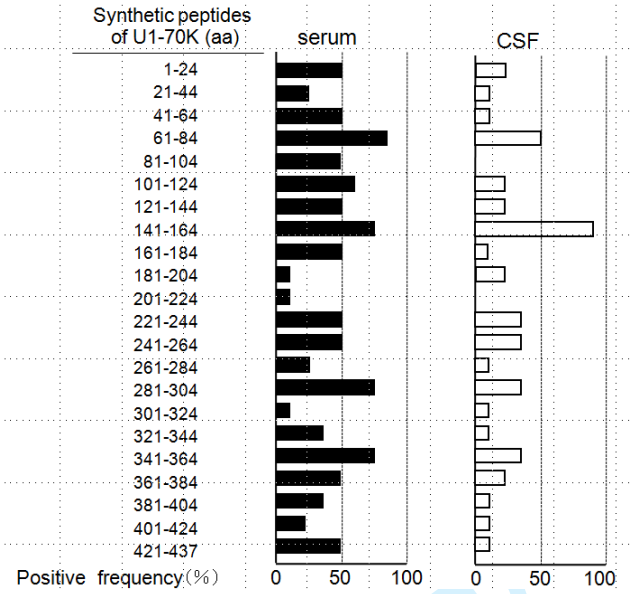

Figure 4. Autoantigenic peptide residues recognized by serum and CSF anti-70K Abs from patients with neuropsychiatric systemic lupus erythematosus (NPSLE). The major autoantigenic domains for serum and CSF anti-U1-70K Abs were examined using ELISA with 22 overlapping peptides identical with the partial sequence of U1-70K (17–24 amino acids [aa]). Sera and CSF from eight patients with central NPSLE were diluted to 100 μg/mL and positivity was determined as more than the mean + 2SD of the negative control samples obtained from patients without serum and CSF anti-U1RNP Abs. The data show the positive frequency of anti-peptide Abs in eight serum and CSF samples from CSF anti-U1RNP Ab-positive patients with central NPSLE.
CSF, cerebrospinal fluid

FIGURE LEGENDS

Figure 1. Antinuclear antibody (ANA) detection in sera and CSF by the RNA immunoprecipitation (RNA-IPP) assay. Anti-U1RNP and anti-SS-A/Ro (SSA) Abs precipitated U1RNA and SSA-RNA, respectively. CNS manifestations are listed in Table 1 and 2. (**A**) Serum and CSF samples from representative patients with central neuropsychiatric systemic lupus erythematosus (NPSLE). (**B**) Serum and CSF samples from representative patients without central NPSLE.

Pt, patient; T, Total RNAs; S, serum; C, cerebrospinal fluid

Figure 2. **(A)** ANA frequency difference in CSF from patients with and without central neuropsychiatric systemic lupus erythematosus (NPSLE). The frequency (%) of CSF ANA-positive in serum ANA-positive patients was shown. Anti-U1RNP and SS-A/Ro Abs were determined by RNA-IPP, and anti-dsDNA Abs were determined by ELISA. Anti-SSB/La and ribosomal P Abs were not detected in CSF. ANA, antinuclear antibodies; CSF, cerebrospinal fluid; IL-6, interleukin-6; Pts, patients; SSA, SS-A/Ro; N.S., not significant. **(B)** Comparison of the IgG and anti-U1RNP ratios. **(C)** Correlations between the

anti-U1RNP index and IL-6, or *Q*alb. **(D)** Correlation between the anti-U1RNP ratio and the IgG index. The Pearson's product moment correlation coefficients (*r*) were calculated in 10 patients with positive CSF anti-U1RNP Abs.

IgG ratio = (CSF IgG concentration [mg/dL]/serum IgG concentration [mg/dL])

Anti-U1RNP ratio (with a compensation for the difference in serum/CSF dilutions) = (100/5) × (CSF anti-U1RNP Ab arbitrary units/serum anti-U1RNP Ab arbitrary units)

*Q*alb (Albumin quotient, normal < 0.0076) = albumin ratio = (CSF albumin concentration [mg/dL]/serum albumin concentration [mg/dL])

IgG index = IgG ratio (CSF IgG concentration [mg/dL]/serum IgG concentration [mg/dL])/*Q*alb

Anti-U1RNP index = (anti-U1RNP ratio/IgG ratio)

Figure 3. Anti-U1RNP index in eight patients with central neuropsychiatric systemic lupus erythematosus (left) and the anti-70K, A, and C indices in the same patients (right) are shown. Serum and cerebrospinal fluid anti-70K, A, and C Ab titers were determined by ELISA using recombinant proteins. The patient number corresponds to the Pt number in

Table 1. Because an insufficient quantity of sample was obtained from Pt.1, the Pt.1 index was excluded from this experiment.

Anti-U1RNP, 70K, A, and C indices (with a compensation for the serum/CSF dilution difference) = (100/5) × ([CSF Ab arbitrary units/serum Ab arbitrary units]/[IgG ratio])

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Figure 4. Autoantigenic peptide residues recognized by serum and CSF anti-70K Abs from patients with neuropsychiatric systemic lupus erythematosus (NPSLE). The major autoantigenic domains for serum and CSF anti-U1-70K Abs were examined using ELISA with 22 overlapping peptides identical with the partial sequence of U1-70K (17–24 amino acids [aa]). Sera and CSF from eight patients with central NPSLE were diluted to 100 μg/mL and positivity was determined as more than the mean + 2SD of the negative control samples obtained from patients without serum and CSF anti-U1RNP Abs. The data show the positive frequency of anti-peptide Abs in eight serum and CSF samples from CSF anti-U1RNP Ab-positive patients with central NPSLE.

CSF, cerebrospinal fluid

Table 1. Patient profiles with central NPSLE

Pt	Age	Diagnosis	CNS manifestations	Cell number in CSF (/μl)	Total protein in CSF (mg/dl)	IL-6 in CSF (pg/ml)	IgG index	*Q*alb ($\times 10^3$)	ANA in serum	ANA in CSF	Anti-U1RNP index	Treatment for CNS manifestations
1	32	SLE	H	0	14	1.0	0.89	1.9	U1, DNA, Sm	U1	13.6	high-dose CS
2	30	SLE	AsM, CgD	5	31	3.2	0.76	3.7	U1, DNA, SSA, Sm, tRNA, riboP	U1, SSA	12.4	mPSL pulse, high-dose CS, CyA, IVCY
3	29	MCTD	AsM	11	81	3240	0.72	15.7	U1, SSA, riboP	U1, SSA	9.4	high-dose CS
4	43	MCTD	Psy	3	49	1.5	0.77	7.2	U1, U2	U1	8.8	mPSL pulse, high-dose CS, IVCY
5	20	SLE	CgD, Psy	0	48	2.3	0.62	8.1	U1, SSA, Sm, riboP	U1	7.6	mPSL pulse, IVCY, DFPP, rituximab
6	40	MCTD	AsM, CgD	40	155	837	0.59	30.3	U1, DNA	U1	3.0	high-dose CS, IVCY
7	22	SLE	ACS, AsM, CVD	32	132	7.8	0.77	20.4	U1, Sm, riboP	U1	2.2	high-dose CS, IVCY
8	32	SLE	AsM	14	128	1190	0.80	18.6	U1, DNA, SSA, SSB	U1, SSA	2.1	mPSL pulse, high-dose CS
9	39	SLE	MovD, H	4	20	1.9	0.61	2.8	U1, SSA	U1	2.1	mPSL pulse, high-dose CS
10	26	SLE	H, Psy	3	29	644	0.83	8.6	U1, SSA	negative	ND	high-dose CS
11	58	SLE	Se, CgD	0	30	2.6	0.55	5.7	U1, SSA	negative	ND	low-dose CS
12	41	SLE	DemS	3	119	ND	0.68	18.6	DNA, SSA	DNA*	ND	high-dose CS, IVCY
13	56	SLE	H, Se	1	35	2.0	0.68	4.5	DNA	negative	ND	mPSL pulse
14	29	SLE	ACS, Psy	1	52	ND	0.65	9.5	negative	negative	ND	high-dose CS, rituximab
		mean±SD		8±13	66±48	494±958	0.71±0.10	11.1±8.3			6.8±4.6	

Pt= patient, CNS= central nervous system, CSF= cerebrospinal fluid, *Q*alb= albumin quotient, ANA= antinuclear antibodies,
SLE= systemic lupus erythematosu, MCTD= mixed connective tissue disease,
H= intractable headache associated with SLE, AsM= aseptic meningitis, CgD= cognitive dysfunction, Psy= psychosis, ACS= acute confusional state, CVD= cerebrovascular disease,
MovD= movement disorder, DemS= demyelinating syndrome, Se= seizures and seizure disorders,
U1= anti-U1RNP Abs, SSA= anti-SS-A/Ro Abs, Sm= anti-Sm Abs, tRNA= anti-transfer RNA Abs, riboP= anti-ribosomal P Abs, U2= anti-U2RNP Abs, DNA= anti-dsDNA Abs,
mPSL= methylprednisolone, CS= corticosteroid, CyA= cyclosporine A, IVCY= intravenous cyclophosphamide, DFPP= double-filtration plasmapheresis,
ND= not determined
*Anti-dsDNA Abs in CSF were determined by ELISA using negative controls (refer to *PATIENTS AND METHODS*).

Table 2. Patient profiles without central NPSLE

Pt	Age at CNS manifestation onset	Diagnosis	CNS manifestations	Cell number in CSF (/μl)	Total protein in CSF (mg/dl)	IL-6 in CSF (pg/ml)	IgG index	Qalb × 10^3	ANA in serum	ANA in CSF	Effective treatment for CNS manifestations
15	29	SLE	AxD	1	31	1.9	1.25	4.3	U1RNP*, DNA, SSA	U1RNP*, SSA	flunitrazepam, cloxazolam
16	19	SLE	H	1	55	20.1	1.38	7.4	U1RNP, DNA, SSA	negative	NSAIDs
17	24	SLE	Se	0	20	1.0	0.59	3.1	U1RNP, DNA, SSA, Sm	negative	sodium valproate
18	28	SLE	CgD	1	22	1.2	0.43	3.6	U1RNP, Sm	negative	diazepam
19	49	SLE	CVD	1	31	ND	0.43	5.6	U1RNP, DNA, Sm	negative	low dose of aspirin
20	22	SLE	CVD	1	36	ND	0.62	5.5	U1RNP, DNA	negative	low dose of aspirin
21	42	MCTD	Drug-induced AsM	10	54	59.1	0.57	12.4	U1RNP, DNA	negative	withdrawal of NSAIDs
22	38	SLE	Miliary tuberculosis	233	214	48300	0.83	31.9	U1RNP, DNA	negative	antitubercular drugs
23	25	SLE	Steroid-induced psychosis	11	31	8.3	0.98	5.6	U1RNP, DNA, SSA	negative	decrease of CS dose
24	32	SLE	Steroid-induced psychosis	1	26	1.4	0.74	4.5	U1RNP, SSA	negative	decrease of CS dose
25	37	SLE	CVD	3	48	ND	0.76	7.6	DNA, SSA	DNA**	low dose of aspirin
26	44	SLE, APS	CVD	4	53	ND	0.57	6.2	DNA, SSA	negative	warfarin
27	31	SLE	H (migraine)	0	21	0.5	ND	ND	DNA	negative	zolmitriptan
28	41	SLE	Steroid-induced psychosis	0	19	0.8	0.80	2.9	SSA	negative	decrease of CS dose
		mean±SD		19±62	47±50	4839±15270	0.77±0.29	7.7±7.7			

Pt= patient, CNS= central nervous system, CSF= cerebrospinal fluid, Qalb= albumin quotient, ANA= antinuclear antibodies, SLE= systemic lupus erythematosus,
MCTD= mixed connective tissue disease, APS= anti-phospholipid Ab syndrome,
AxD= anxiety disorder, H= intractable headache unrelated to SLE, Se= seizures and seizure disorders, CgD= cognitive dysfunction, CVD= cerebrovascular disease,
AsM= aseptic meningitis,
U1= anti-U1RNP Abs, SSA= anti-SS-A/Ro Abs, Sm= anti-Sm Abs, tRNA= anti-transfer RNA Abs, riboP= anti-ribosomal P Abs, U2= anti-U2RNP Abs, DNA= anti-dsDNA Abs,
NSAIDs= non-steroidal anti-inflammatory drugs, CS= corticosteroid.
ND= not determined
*anti-U1RNP index = 4.7
**Anti-dsDNA Abs in CSF were determined by ELISA using negative controls (refer to *PATIENTS AND METHODS*).

Full-length article (clean copy)

Anti-U1 RNP antibodies in cerebrospinal fluid are associated with central neuropsychiatric manifestations in systemic lupus erythematosus and mixed connective tissue disease

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Objective. To determine the significance of anti-U1 ribonucleoprotein (RNP) antibodies (Abs) in cerebrospinal fluid (CSF) from systemic lupus erythematosus (SLE) and mixed connective tissue disease (MCTD) patients with central neuropsychiatric (NP) SLE.

Methods. Antinuclear Abs including anti-U1RNP Abs were determined in sera and CSF of 24 patients with SLE and four patients with MCTD and central NPSLE using an RNA immunoprecipitation assay and ELISA. The frequency of CSF anti-U1RNP Abs in patients with central NPSLE was examined, and the anti-U1RNP index (= [CSF anti-U1RNP Abs /serum anti-U1RNP Abs] / [CSF IgG /serum IgG]) was compared with CSF interleukin (IL)-6 levels and the albumin quotient (*Q*alb, an indicator of blood brain barrier damage). CSF and serum Abs against U1-70K, A, and C including autoantigenic regions were examined, and the anti-70K, A, and C indices as well as the anti-U1RNP index were calculated.

Results. CSF anti-U1RNP Abs with an increased anti-U1RNP index showed 64% sensitivity and 93% specificity for central NPSLE. The anti-U1RNP index did not correlate with CSF IL-6 levels or *Q*alb. The anti-70K index was higher than the anti-A and C indices in CSF of anti-U1RNP Ab-positive patients with central NPSLE. The major autoantigenic

region for CSF anti-70K Abs appeared to be localized in the U1-70K 141–164 amino acid residues within the RNA-binding domain.

Conclusions. CSF anti-U1RNP Abs and the anti-U1RNP index are useful indicators for central NPSLE in anti-U1RNP Ab-positive patients. The predominance of anti-70K Abs in CSF suggests intrathecal anti-U1RNP Ab production.

Systemic lupus erythematosus (SLE) is a chronic, remitting and relapsing, multisystem autoimmune disease that predominantly affects women. Among various organ damages, neuropsychiatric SLE involving the central nervous system (CNS; central NPSLE) is a life-threatening severe manifestation. The overall prevalence of NPSLE varies between 14 and 75% in a representative selection of studies using the American College of Rheumatology (ACR) nomenclature (1). In fact, NPSLE may affect mortality in SLE and is associated with a significant negative impact on a patients' quality of life. However, 41% of all CNS manifestations in SLE patients may be attributed to factors other than lupus (2).

Although the pathophysiology of most NPSLE cases is not well determined, a particular subset of autoantibodies (Abs) is associated with neuronal injury. A subset of anti-DNA Abs cross-reacts with a sequence within the *N*-methyl-D-aspartate receptor subunit NR2 (anti-NR2 Abs), causing excitatory synaptic transmission in the CNS (3). Anti-NR2 Abs preferentially target the hippocampus, suggesting that anti-NR2 Abs may disrupt normal cognitive processes (4). However, serum anti-NR2 Abs may be associated with depressive mood (5), but not cognitive dysfunction (5, 6). Arinuma et al. (7) reported that anti-NR2 Abs in cerebrospinal fluid (CSF), but not in serum, were involved in diffuse central NPSLE.

Anti-ribosomal P Abs have been reported as neurotoxic autoAbs (8–10). Abnormal behavior can be induced in normal mice by administering anti-ribosomal P Abs (10), although serum anti-ribosomal P Abs are not specific to human central NPSLE (11). In association with thrombosis, anti-β2-glycoprotein I and prothrombin Abs are recognized as pathogenic Abs in focal central NPSLE such as cerebrovascular disease (12).

In addition to autoAbs, several proinflammatory cytokines and chemokines have been detected in serum and CSF of patients with SLE. The most important cytokine in SLE and NPSLE may be interferon (IFN)-α (13–15). A recent report showed the presence of IFN-α, IFN-γ-inducible protein 10 (IP-10), interleukin (IL)-8, and monocyte chemotactic protein (MCP)-1 in the CSF from patients with NPSLE (15). IFN-α generation in SLE is caused, at least partially, by autoAbs that bind to ribonucleoprotein (RNP) particles released from dead and dying cells. Santer et al. clearly suggested that IFN-inducing activity in CSF correlates with serum anti-U1RNP Abs, but not with other known antinuclear antibodies (ANA) (15). Therefore, anti-U1RNP Abs and their immune complex in CSF may have pathogenic roles in central NPSLE. Okada et al. reported 14 patients with aseptic meningitis (eight with SLE, six with mixed connective tissue disease [MCTD] or undifferentiated

connective tissue disease, and one with Sjögren's syndrome) among 1560 patients with connective tissue disease. Serum anti-U1RNP Abs were positive in 13 of the 14 patients with aseptic meningitis, suggesting that anti-U1RNP Abs may be linked to central NP manifestations (16). In the present study, we evaluated the clinical significance and immunological characteristics of anti-U1RNP Abs in CSF derived from SLE and MCTD patients with central NP manifestations.

PATIENTS AND METHODS

Patients. Patients with SLE or MCTD who revealed CNS manifestations and had been admitted to the Department of Rheumatology and Clinical Immunology, Kyoto University Hospital, from March 2002 to October 2007 were enrolled. SLE was diagnosed according to the ACR criteria (17, 18) and MCTD was diagnosed according to the criteria proposed by the Ministry of Health and Welfare in Japan (19). CNS manifestations were classified according to the case definitions for NP syndromes in SLE (20). Evaluation of NP syndromes included neuropsychiatric testing and magnetic resonance imaging (MRI) of the brain. Patients were diagnosed with central NPSLE retrospectively, according to

Kwiecinski's criteria (21) with some modifications. Briefly, central NPSLE was defined as the presence of at least two of the following six items: 1) recent-onset of psychosis, 2) transverse myelitis, 3) aseptic meningitis, 4) seizures, 5) pathologic changes visualized on brain MRI, and 6) severely abnormal cognitive dysfunction documented by neuropsychiatric testing. Oligoclonal IgG bands in the CSF were not included, and CNS manifestations caused by other factors (e.g., concurrent non-SLE NP disease such as infection and secondary NPSLE such as uremia, hypertension, and complications of SLE therapy) were not defined as central NPSLE. In the present study, small punctate focal lesions in white matter, cortical atrophy, periventricular white matter hyperintensity, diffuse white matter changes, discrete gray matter lesions, diffuse gray matter hyperintensities, cerebral edema, and new infarct were considered as pathologic changes on brain MRI. The present study was conducted in compliance with the Declaration of Helsinki and approved by the Kyoto University Ethics Committee Review Board (approval #E97).

Samples. After an acute phase of massive cerebrovascular disease (CVD) was ruled out by brain MRI screening, CSF was taken from patients within 3 days from the onset of CNS manifestations. We have a written informed consent from all of the studied patients.

Serum samples were collected from all patients on the same day, and both samples were stored at -80°C. Routine CSF and serum analyses, including total protein, albumin, and IgG level, were conducted. CSF interleukin (IL)-6 (R&D Systems, Minneapolis, MN, USA) and interferon (IFN)-α (Bender MedSystems, Vienna, Austria) levels were determined by ELISA, according to the manufacturer's protocol. The sensitivity of the IFN-α assay was more than 5 pg/mL.

***Q*alb and IgG index.** To determine blood brain barrier (BBB) damage, the albumin quotient (*Q*alb, normal < 0.0076) was calculated as the ratio of CSF albumin (mg/dL) to serum albumin (mg/dL) (22). The IgG index was calculated as the IgG ratio (CSF IgG concentration [mg/dL] /serum IgG concentration [mg/dL])/*Q*alb.

Detection of antinuclear antibodies (ANA) in sera and CSF by RNA immunoprecipitation. RNA immunoprecipitation (RNA-IPP) using HeLa cell extracts was performed to determine anti-RNA-associated antigen Abs, such as anti-U1RNP, Sm, SS-A/Ro (SSA), SS-B/La (SSB), and ribosomal P Abs in sera and CSF (23). A 10 µl sample of sera or CSF was mixed with 2 mg of protein A Sepharose™ CL-4B (GE Healthcare, Upsala, Sweden) in 500 µl of IPP buffer (10 mM Tris-HCl at pH 8.0, 500 mM NaCl, 0.1%

Nonidet P-40 [NP-40]) and incubated on a rotator for 2 h at 4°C. The IgG-coated Sepharose was washed four times in 500 µl of IPP buffer using 10-s spins in a microfuge and resuspended in 400 µl of NET-2 buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.05% NP-40). For RNA analysis, this suspension was incubated with 300 µl of HeLa cell extracts, derived from 6×10^6 cells, on a rotator for 2 h at 4°C. The antigen-bound Sepharose was then collected by a 10-s centrifugation in a microfuge, washed four times with 500 µl of NET-2 buffer, and resuspended in 270 ml of NET-2 buffer. To extract bound RNAs, 30 µl of 3.0 M sodium acetate, 15 µl of 20% sodium dodecyl sulfate, and 300 µl of phenol/chloroform/isoamyl alcohol (50:50:1; containing 0.2 g of 8-hydroxyquinoline and 40 ml of 0.1 M Tris-HCl at pH 7.5) were added to the Sepharose beads. After agitation in a vortex mixer and spinning for 1 min, RNAs were recovered in the aqueous phase after ethanol precipitation and dissolved in 20 µl of electrophoresis sample buffer composed of 10 M urea, 0.025% bromphenol blue, and 0.02 % xylene cyanol FF (Bio-Rad, Hercules, CA, USA) in Tris-borate-EDTA buffer (90 mM Tris HCl at pH 8.6, 90 mM boric acid, and 1 mM EDTA). The RNA samples were denatured at 65°C for 5 min and then resolved by 7 M urea-10% polyacrylamide gel electrophoresis with silver staining (Bio-Rad). Anti-U1RNP,

Sm, SSA, and SSB Abs were determined as positive when U1RNA, U1-U6RNA, SSA-RNA (Y1-Y5RNA), and SSB-RNA (5S-ribosomal RNA, 7S-RNA, and Y1-Y5RNA) were precipitated, respectively.

Detection of ANA in sera and CSF by ELISA. Serum anti-U1RNP, dsDNA, SSA, and ribosomal P Abs were also determined by ELISA using recombinant U1RNP (Mesacup®-2 test RNP, Medical and Biological Laboratories [MBL] Co., Nagoya, Japan), purified dsDNA (Mesacup DNA-II test ds®, MBL Co.), recombinant SS-A (Mesacup®-2 test SS-A, MBL Co.), and recombinant ribosomal P proteins (Ribosomal P ELISA kit, MBL Co.), according to the manufacturer's protocol. Anti-ribosomal P and dsDNA Abs in CSF were determined to be positive when the experimental titer was more than that of the mean + 2SD of 10 negative controls. CSF samples derived from patients with other autoimmuine diseases (4 with polyarteritis nodosa, 4 with multiple sclerosis, and 2 with rheumatoid arthritis) were used as negative CSF controls for the anti-ribosomal P and dsDNA Abs. These patients had CNS manifestation and/or CSF abnormality, but not NPSLE, and negative results for their serum ANA were confirmed. Sera were diluted 1:100, and CSF samples were diluted 1:5 using phosphate buffer saline (PBS). To determine the levels of anti-70K, A, and C Abs,

recombinant U1-70K, A, and C (MBL Co.) along with coating buffer (pH 9.4) (1 μg/mL) were bound to ELISA plates, and the wells were blocked using 5% bovine serum albumin (BSA) in PBS. Patient sera and CSF containing anti-U1RNP Abs were added and incubated at room temperature for 2 h, followed by detection of bound IgG with alkaline phosphatase-conjugated anti-human IgG (Southern Biotechnology Associates Inc., Birmingham, AL, USA) at $OD_{405\ nm}$ in a microtiter ELISA reader. All assays were performed in triplicate. We confirmed that the OD_{405nm} values of the CSF-experimental wells were included within the linear range of the positive control. If a 1:5 dilution was not appropriate, the dilution was changed in such samples and the obtained titer was adjusted.

Anti-U1RNP ratio and anti-U1RNP index. Arbitrary units of the anti-U1RNP Abs in each sample were determined using serum and CSF anti-U1RNP Ab-positive (defined as 100 units) and negative standard samples. More precisely, the arbitrary units for serum or CSF anti-U1RNP Abs were calculated as: ([$OD_{405\ nm}$ of experimental well − $OD_{405\ nm}$ of anti-U1RNP Ab-negative standard well] × 100/[$OD_{405\ nm}$ of anti-U1RNP Ab-positive standard well − $OD_{405\ nm}$ of anti-U1RNP Ab-negative standard well]). After obtaining the arbitrary units of the anti-U1RNP Ab, the anti-U1RNP ratio and anti-U1RNP index were

calculated as: (CSF arbitrary anti-U1RNP Ab units × 5/serum arbitrary anti-U1RNP Ab units × 100) and anti-U1RNP ratio/IgG ratio, respectively. The difference in the dilutions between serum (×100) and CSF (×5) was adjusted, and the anti-70K, A, and C indices were obtained by the same calculation method.

Determination of autoantigenic regions recognized by serum and CSF anti-70K Abs. To compare the autoantigenic regions in the U1-70K proteins recognized by the CSF and serum anti-U1RNP Abs, we prepared 22 overlapping synthetic peptides (17–24 amino acids [aa]) identical with the U1-70K partial sequences (24) (Fig. 4). Synthetic peptides (100 μg/ml) with coating buffer (pH 9.4) were bound to ELISA plates and the wells were blocked with 5% BSA in PBS. Sera and CSF from eight patients with central NPSLE were diluted to 100 μg/mL with PBS and then incubated for 2 h at room temperature, followed by detection of bound IgG with alkaline phosphatase-conjugated anti-human IgG (Southern Biotechnology Associates Inc.) at $OD_{405\ nm}$ in a microtiter ELISA reader. All assays were performed in triplicate. Positivity was determined as more than the mean + 2SD of the negative control samples obtained from patients without serum and CSF anti-U1RNP Abs.

Statistical analysis. Differences in the frequencies of ANA in CSF and serum were

evaluated by the chi-square (χ^2) test and Fisher's exact test as appropriate. The Student's *t*-test was used to compare the difference between two group means. Pearson's product moment correlation coefficients (*r*) were calculated to evaluate the correlation between the anti-U1RNP Ab index and other indicators. A *p* value less than 0.05 was considered significant.

RESULTS

Patient characteristics. NP syndromes were detected in 24 patients with SLE and four patients with MCTD. All patients were female, and their age at onset of CNS manifestations was 34.1 years (range, 19–58 years). According to the ACR nomenclature (20), the symptoms of central NPSLE exhibited by our patients were as follows: headache, cerebrovascular disease, cognitive dysfunction, seizures and seizure disorder, aseptic meningitis, psychosis, acute confusional state, demyelinating syndrome, anxiety disorder, and movement disorder.

CSF findings and ANA profiles in patients with central NPSLE. Fourteen patients were diagnosed with central NPSLE (Table 1). All patients except for patient 11 needed a

high-dose corticosteroid treatment along with methylprednisolone pulse therapy and/or immunosuppressive agents for their central NPSLE. Aseptic meningitis most frequently occurred (five patients). Of the 14 patients, the cell number in CSF increased in six patients (46%, normal < 4 cells/μl), and total protein concentration increased in eight patients (57%, normal < 40 mg/dL). CSF IL-6 was elevated in five of 12 patients (42%, normal < 4.3 pg/mL [25]), and the IgG index increased in nine of 14 (64%, normal < 0.67). *Q*alb, an index of blood brain barrier (BBB) permeability, increased in eight of 14 patients (57%, normal < 0.0076 [22]). By RNA-IPP, anti-U1RNP, SSA, SSB, and Sm Abs were detected in sera from 11 (79%), eight (57%), one (7%), and four (29%) patients, respectively. Anti-ribosomal P and dsDNA Abs were determined as positive by ELISA in sera from four (29%) and six (43%) patients, respectively. In contrast, anti-U1RNP Abs in CSF were most frequently detected by RNA-IPP (82%) in CSF from anti-U1RNP Ab-positive patients with central NPSLE (Fig. 1 and 2A). The anti-U1RNP ratios increased more than the IgG ratios in patients with CSF-anti-U1RNP Abs (Fig. 2B). CSF anti-SSA and dsDNA Abs were detected in only three and one patient, respectively. Anti-ribosomal P and Sm Abs were absent in CSF.

CSF findings and ANA profiles in patients without central NPSLE. CNS

manifestations were diagnosed in 14 patients as concurrent non-SLE central NP diseases or secondary central NP syndromes (Table 2). Increased cell number and protein concentrations were observed in three (21%) and five (36%) patients, respectively. IL-6, the IgG index, and *Q*alb were elevated in four (40%), seven (54%), and two (15%) out of 14 patients, respectively. These values were not significantly different between patients with and without central NPSLE. In contrast to central NPSLE, CSF anti-U1RNP Abs were detected in only one patient (#15) without central NPSLE (Fig. 2A), whereas serum anti-U1RNP Abs were detected frequently (71%).

Correlation of anti-U1RNP index to CSF IL-6 level and *Q*alb. The anti-U1RNP index was compared to CSF IL-6 and *Q*alb determined from the same samples in 10 CSF anti-U1RNP Ab-positive patients (# 1–9, and 15). The anti-U1RNP index was independent of the CSF IL-6 (Fig. 2C) and anti-U1RNP ratio was not correlated to the IgG indices (Fig. 2D). *Q*alb, which is reportedly a useful BBB permeability indicator, tended to inversely correlate with the anti-U1RNP index (Fig. 2C, $y = -0.246x + 9.37$).

Anti-70K Abs were most dominant in CSF among the Abs against U1RNP-specific proteins. Anti-U1RNP Abs usually recognize either U1-70K, A, or C

proteins, which are the unique components of the U1RNP particle. We examined the anti-70K, A, and C indices as well as the anti-U1RNP index in eight CSF anti-U1RNP Ab-positive patients with central NPSLE (refer to *Table 1*), and the anti-U1RNP index was greater than 2.0 in all patients. In the same samples, there was an elevation of the anti-70K index (> 1.0) in seven of eight patients, whereas the anti-A and C indices were elevated in only a few patients. The average anti-70K index was significantly higher than that of the anti-C index and tended to be higher than that of the anti-A index (Fig. 3).

U1-70K autoantigenic regions recognized by serum and CSF anti-U1RNP Abs. More detailed reactivity of the U1-70K protein was examined in sera and CSF from eight patients with CSF anti-U1RNP Abs. All prepared synthetic peptides were recognized by the eight anti-U1RNP Ab-positive sera, whereas there were 4–21 reacted residues for each serum sample (data not shown). Serum anti-U1RNP Abs in patients with central NPSLE bound the 61–84 aa residue most frequently (88%) (Fig. 4). In contrast, the synthetic peptides that bound CSF anti-U1RNP Abs were limited to 20 residues, and 1–12 was the reacted residue number for each CSF sample (data not shown). The 141–164 aa residue alone was recognized by the majority of the CSF anti-U1RNP Abs (88%), whereas autoepitope pattern

recognized by serum- and CSF-anti-U1RNP Abs was not significantly different (Fig. 4).

DISCUSSION

We showed that CSF anti-U1RNP Abs determined by RNA-IPP and/or an elevation in the anti-U1RNP index are more specific markers for central NPSLE than the CSF IL-6 and IgG indices in serum anti-U1RNP Ab-positive SLE and MCTD patients. This is the first report showing the clinical significance of CSF anti-U1RNP Abs. While other autoAbs such as anti-ribosomal P, NR2, or dsDNA Abs in association with NPSLE have been described, there is no report regarding serum or CSF anti-U1RNP Abs in an international cohort or large study (26, 27). Recently, the sensitivity and specificity of CSF IL-6 (>4.3 pg/mL) for diagnosing central NPSLE have been reported as 87.5% and 92.3%, respectively (25). However, CSF IL-6 is not specific for disease-associated NPSLE because an IL-6 elevation can also be caused by infectious meningoencephalitis and cerebrovascular disease. The IgG index is also elevated in patients without CNS involvement (28), and no statistical differences in IL-6 and the IgG index were observed between central NPSLE and other CNS manifestations in the present study. *Q*alb elevation is strong evidence for BBB damage (22),

and more than half of our patients with central NPSLE had increased BBB permeability, similar to that reported in a previous study (29). However, the BBB damage is caused not only by central NPSLE but also by other factors. *Q*alb was elevated in patients with drug-induced aseptic meningitis (# 21) and miliary tuberculosis (# 22). The presence of anti-U1RNP Abs in CSF along with an anti-U1RNP index of more than 2.0 was frequently observed in patients with, but not without, central NPSLE (sensitivity = 64.3%, specificity = 92.9%). Moreover, the sensitivity and specificity of CSF anti-U1RNP Abs for central NPSLE in serum anti-U1RNP Ab-positive patients were calculated as 81.8% and 90.0%, respectively, While global penetration of serum Abs into CSF occurs from a serious BBB injury, elevation of the anti-U1RNP index is not influenced, because the IgG ratio increases simultaneously in this condition. Thus, the increased anti-U1RNP Ab titer is a possible diagnostic marker for CNS manifestations attributable to SLE or MCTD independent of CSF IL-6 level, IgG index, or *Q*alb.

In a previous study, a correlation was observed between serum anti-Sm Abs and central NPSLE (30), whereas the present study demonstrated no association between the presence of serum anti-U1RNP/Sm Abs and central NPSLE. To date, only our group (31)

and a German group (32) have published case reports of CSF anti-U1RNP Ab-positive patients. The important point of our study may be the use of RNA-IPP for detecting anti-U1RNP Abs. RNA-IPP is the most sensitive and specific method among the immunological methods used for detecting Abs, especially against RNA or RNA-binding proteins (33). The absence of anti-Sm Abs may not be due to a sensitivity problem with the RNA-IPP, because we could not detect the Abs in the same samples by ELISA. Because of the lower amount of cellular SSA-RNAs than U1RNAs, SSA-RNAs are more difficult to visualize than U1RNAs by RNA-IPP. It is possible that anti-U1RNP Abs were unequivocally detected in this system, and the present study may not indicate that an intrathecal ANA stimulation is specific to anti-U1RNP Abs.

The striking deficit in CNS pathology, specifically the lack of vasculitis or massive cellular infiltrate in patients dying of central NPSLE, suggests that the pathogenesis differs from immune complex (IC) deposition, which is a characteristic of lupus nephritis. Rather, anti-U1RNP Abs may act as an inducer of proinflammatory cytokines. It is worth noting that serum and CSF of patients with NPSLE show abnormally high IFN-α-inducing activity (15). In addition to IFN-α and IC formed by CSF, autoAbs produce significantly increased levels

of IFN-γ-inducible protein 10 (IP-10/CXCL), IL-8, and monocyte chemotactic protein-1 (34-36), and this phenomenon is most distinguished in the serum of anti-U1RNP Ab-positive patients (15). It is interesting that this hypothesis was demonstrated, because IFN-α is strongly induced by U1RNP-containing IC (37) and is the key cytokine for SLE pathogenesis. IC and IFN-α were not detected in most of our CSF samples (data not shown), possibly because CSF IFN-α was disrupted quickly and IC moved to FcγR-expressing cells.

Thus far, it has been reported that a certain ANA subset is relevant to central NPSLE. First, serum anti-ribosomal P Abs are definitely a useful diagnostic marker for SLE (38); however, it is controversial whether anti-ribosomal P Abs in serum and/or CSF are a link to central NPSLE or not (11, 38), whereas the presence of ribosomal P protein on the endothelial cells has been demonstrated (39). It is likely that anti-ribosomal P Abs might not be able to pass effectively through the BBB due to binding to CNS endothelial cells (8). When paired serum and CSF samples were diluted to the same IgG concentrations and used for Western blotting, selective enrichment of IgG anti-ribosomal P occurred in the CSF of a few patients (39). Anti-ribosomal P Abs were not detected in CSF from our patients. Second, Kowal et al. have reported that a certain anti-dsDNA Ab subset in CSF, which cross-reacts

with the NR2 glutamate receptor, causes apoptotic neuronal death in the mouse hippocampus (3). Although the CSF level of anti-NR2 Abs was higher in patients with central NPSLE than that in other SLE groups, the highest CSF anti-NR2 Ab levels have been detected in patients with septic meningitis (26), suggesting that anti-NR2 Abs attack neurotransmitters directly through a breach in BBB integrity. While serum and CSF anti-NR2 Abs were not investigated in the present study, the anti-NR2 Ab-mediated neuronal diseases are different than aseptic meningitis and appear to be associated with anti-U1RNP Abs. Unfortunately, the present study did not clearly indicate which types of central NPSLE that anti-U1RNP Abs are most associated with. In accordance with a previous study (27), our data show no significant correlation between the presence of serum- or CSF-anti-SSA Abs and central NPSLE.

The presence of ANA in CSF can be explained by three mechanisms: (1) in situ Ab production in the CNS, (2) a BBB breach, which would allow Abs to cross a normally restricted compartment, and (3) an increased Ab concentration resulting from a reduced CSF flow rate. However, our data strongly suggest intrathecal production of anti-U1 RNP Abs, because the anti-70K, A, and C indices were not equally elevated, and the anti-U1RNP and

70K indices in most patients increased by more than 2.0. Even if a BBB breach or reduced flow occurred, it is unlikely that either anti-70K, A, or C Abs penetrated or moved outside the CSF. The observation that a different U1-70K peptide recognition pattern by serum and CSF anti-70K Abs is evident also suggests intrathecal anti-U1RNP Ab production. Remarkably, the autoantigenic 141–164 aa residue for CSF anti-70K Abs in patients with central NPSLE was located within the RNA-binding domain (92–202 aa), including T (40) and B (41–44) cell major epitopes of human anti-70K Abs. Guldner et al. identified the 56–195 aa domain as the major antigenic epitope recognized by all tested sera (41). Cram et al. reported the 100–156 aa residue as one of the major epitopes in the human 70K protein (42). James et al. showed that the basic aa-rich sequences are the early autoantigenic determinants of the 70K C-terminus (45). More detailed experiments using a large number of CSF samples are necessary to clarify the immunological characteristics of intrathecal anti-U1RNP Abs, whereas our results suggest the possibility that an ANA production in CSF is stimulated by an antigen-driven mechanism.

In conclusion, CSF anti-U1RNP Abs, which may be produced in the CNS, are a clinically useful indicator for central NPSLE. However, there are some limitations to the

present study. First, the usefulness of the anti-U1RNP index is limited to patients with NPSLE that have serum anti-U1RNP Abs. Second, RNA-IPP method may be required to determine a low anti-U1RNP Ab titer, and third, our results were observed in a small number of Japanese patients. A more detailed association of CSF anti-U1RNP Abs with other humoral factors or activated neuronal cells in the CNS should be elucidated in a future study.

AUTHOR CONTRIBUTIONS

Dr. Fujii had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design.	Fujii, Mimori
Patient recruitment.	Imura, Yukawa, Kawabata, Nojima, Ohmura, Usui
Acquisition of data.	Sato, Yokoyama
Manuscript preparation.	Sato, Fujii
Statistical analysis.	Sato, Fujita
Interpretation of data.	Mimori

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FIGURES

Figure 1.

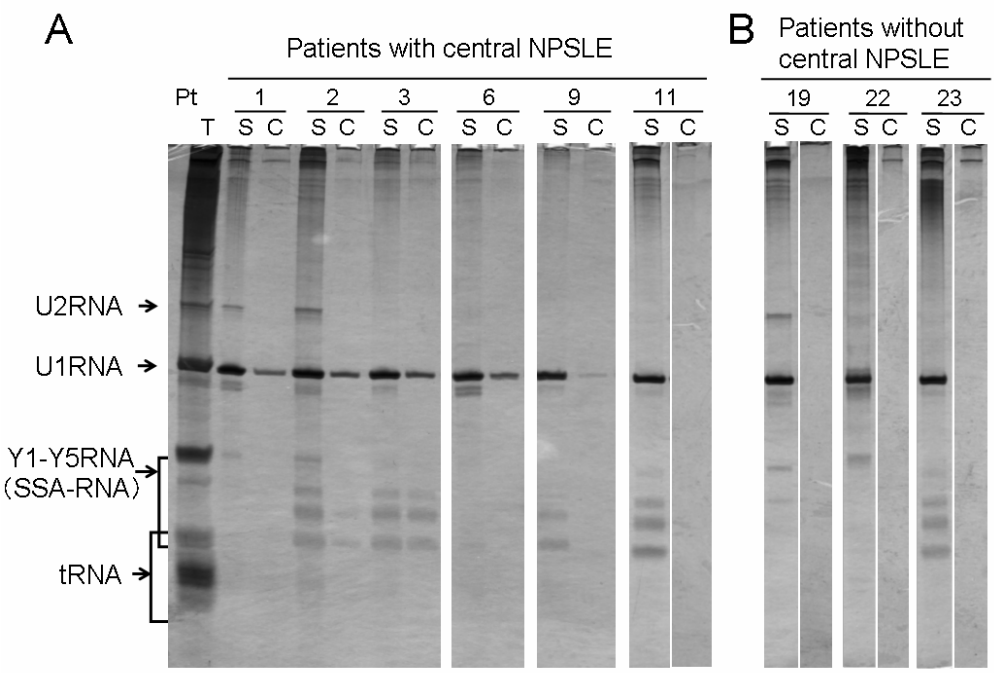

Figure 1. Antinuclear antibody (ANA) detection in sera and CSF by the RNA immunoprecipitation (RNA-IPP) assay. Anti-U1RNP and anti-SS-A/Ro (SSA) Abs precipitated U1RNA and SSA-RNA, respectively. CNS manifestations are listed in Table 1 and 2. (**A**) Serum and CSF samples from representative patients with central neuropsychiatric systemic lupus erythematosus (NPSLE). (**B**) Serum and CSF samples from representative patients without central NPSLE.

Figure 2.

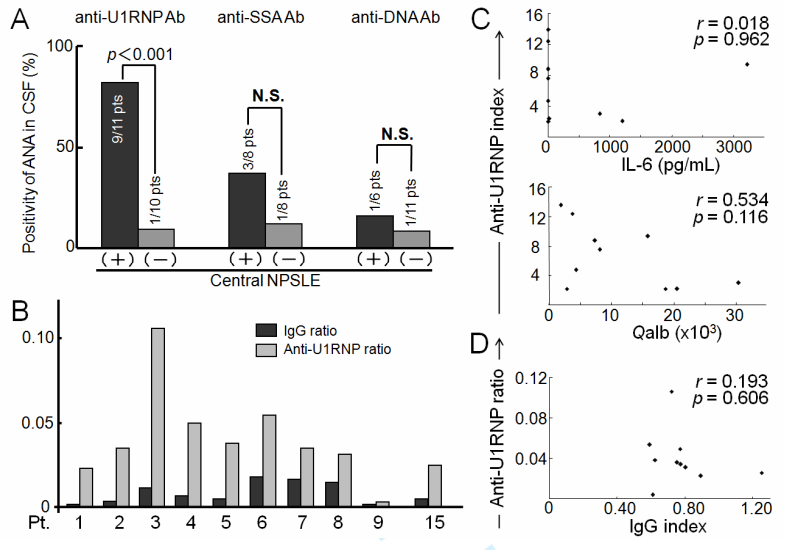

Figure 2. **(A)** ANA frequency difference in CSF from patients with and without central neuropsychiatric systemic lupus erythematosus (NPSLE). The frequency (%) of CSF ANA-positive in serum ANA-positive patients was shown. Anti-U1RNP and SS-A/Ro Abs were determined by RNA-IPP, and anti-dsDNA Abs were determined by ELISA. Anti-SSB/La and ribosomal P Abs were not detected in CSF. ANA, antinuclear antibodies; CSF, cerebrospinal fluid; IL-6, interleukin-6; Pts, patients; SSA, SS-A/Ro; N.S., not significant. **(B)** Comparison of the IgG and anti-U1RNP ratios. **(C)** Correlations between the anti-U1RNP index and IL-6, or *Q*alb. **(D)** Correlation between the anti-U1RNP ratio and the IgG index. The Pearson's product moment correlation coefficients (r) were calculated in 10 patients with positive CSF anti-U1RNP Abs. IgG ratio = (CSF IgG concentration [mg/dL]/serum IgG concentration [mg/dL]), Anti-U1RNP ratio (with a compensation for the difference in serum/CSF dilutions) = (100/5) × (CSF anti-U1RNP Ab arbitrary units/serum anti-U1RNP Ab arbitrary units), *Q*alb (Albumin quotient, normal < 0.0076) = albumin ratio = (CSF albumin concentration [mg/dL]/serum albumin concentration [mg/dL]), IgG index = IgG ratio (CSF IgG concentration [mg/dL]/serum IgG concentration [mg/dL])/*Q*alb, Anti-U1RNP index = (anti-U1RNP ratio/IgG ratio)

Figure 3.

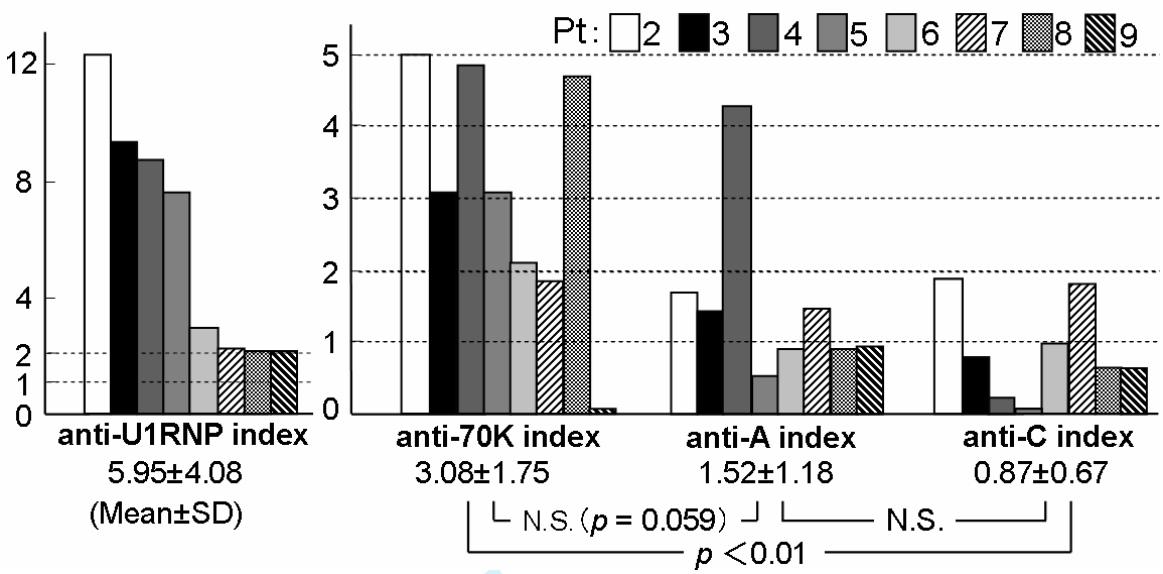

Figure 3. Anti-U1RNP index in eight patients with central neuropsychiatric systemic lupus erythematosus (left) and the anti-70K, A, and C indices in the same patients (right) are shown. Serum and cerebrospinal fluid anti-70K, A, and C Ab titers were determined by ELISA using recombinant proteins. The patient number corresponds to the Pt number in Table 1. Because an insufficient quantity of sample was obtained from Pt.1, the Pt.1 index was excluded from this experiment.
Anti-U1RNP, 70K, A, and C indices (with a compensation for the serum/CSF dilution difference) = (100/5) × ([CSF Ab arbitrary units/serum Ab arbitrary units]/[IgG ratio])
Pt, patient; N.S., not significant

Figure 4.

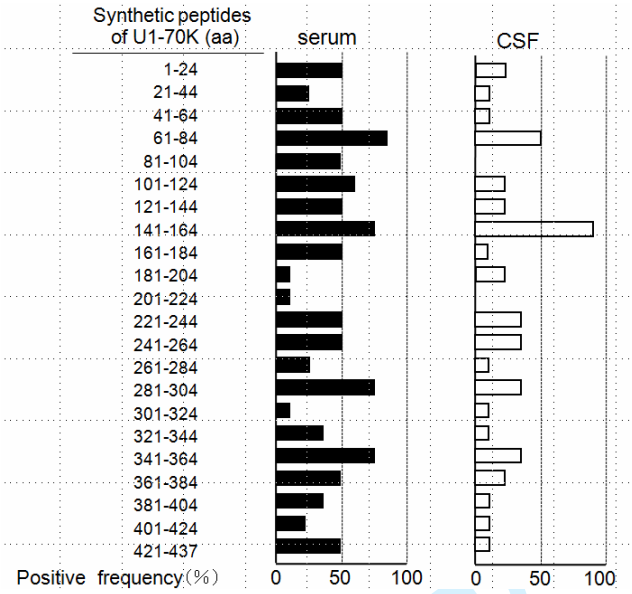

Figure 4. Autoantigenic peptide residues recognized by serum and CSF anti-70K Abs from patients with neuropsychiatric systemic lupus erythematosus (NPSLE). The major autoantigenic domains for serum and CSF anti-U1-70K Abs were examined using ELISA with 22 overlapping peptides identical with the partial sequence of U1-70K (17–24 amino acids [aa]). Sera and CSF from eight patients with central NPSLE were diluted to 100 μg/mL and positivity was determined as more than the mean + 2SD of the negative control samples obtained from patients without serum and CSF anti-U1RNP Abs. The data show the positive frequency of anti-peptide Abs in eight serum and CSF samples from CSF anti-U1RNP Ab-positive patients with central NPSLE.
CSF, cerebrospinal fluid

FIGURE LEGENDS

Figure 1. Antinuclear antibody (ANA) detection in sera and CSF by the RNA immunoprecipitation (RNA-IPP) assay. Anti-U1RNP and anti-SS-A/Ro (SSA) Abs precipitated U1RNA and SSA-RNA, respectively. CNS manifestations are listed in Table 1 and 2. (**A**) Serum and CSF samples from representative patients with central neuropsychiatric systemic lupus erythematosus (NPSLE). (**B**) Serum and CSF samples from representative patients without central NPSLE.

Pt, patient; T, Total RNAs; S, serum; C, cerebrospinal fluid

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IgG ratio = (CSF IgG concentration [mg/dL]/serum IgG concentration [mg/dL])

Anti-U1RNP ratio (with a compensation for the difference in serum/CSF dilutions) = (100/5) × (CSF anti-U1RNP Ab arbitrary units/serum anti-U1RNP Ab arbitrary units)

*Q*alb (Albumin quotient, normal < 0.0076) = albumin ratio = (CSF albumin concentration [mg/dL]/serum albumin concentration [mg/dL])

IgG index = IgG ratio (CSF IgG concentration [mg/dL]/serum IgG concentration [mg/dL])/*Q*alb

Anti-U1RNP index = (anti-U1RNP ratio/IgG ratio)

Figure 3. Anti-U1RNP index in eight patients with central neuropsychiatric systemic lupus erythematosus (left) and the anti-70K, A, and C indices in the same patients (right) are shown. Serum and cerebrospinal fluid anti-70K, A, and C Ab titers were determined by ELISA using recombinant proteins. The patient number corresponds to the Pt number in

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