Identification of Three New Autoantibodies Associated with Systemic Lupus Erythematosus Using Two Proteomic Approaches

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**Abbreviations:**

SLE: systemic lupus erythematosus  
CNS: central nervous system  
NP: neuropsychiatric  
ACR: American College of Rheumatology  
RA: rheumatoid arthritis  
SSc: systemic sclerosis  
SS: Sjögren syndrome  
MS: multiple sclerosis  
BCA: bicinchoninic acid  
WB: western blot  
HRP: horseradish peroxidase  
CBB: Coomassie Brilliant Blue  
CHCA: α-cyano-4-hydroxycinnamic acid  
DSS: disuccinimidyl suberate  
NHC: normal healthy control
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SUMMARY
Our objective was to identify new serum autoantibodies associated with systemic lupus erythematosus (SLE), focusing on those found in patients with central nervous system (CNS) syndromes. Autoantigens in human brain proteins were screened by multiple proteomic analyses: 2-dimensional polyacrylamide gel electrophoresis/western blots followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis and immunoprecipitation followed by liquid chromatography-tandem mass spectrometry shotgun analysis. The presence of serum IgG autoantibodies against 11 selected recombinant antigens was assessed by western blot and enzyme-linked immunosorbent assay (ELISA) in the sera of 106 SLE patients and 100 normal healthy controls. The O.D. values in sera from SLE patients were significantly higher than those of controls for the antigens crystallin αB (p=0.0002), esterase D (p=0.0002), APEX nuclease 1 (p < 0.0001), ribosomal protein P0 (p < 0.0001), and PA28γ (p=0.0005); the first three are newly reported. The anti-esterase D antibody levels were significantly higher in the CNS group than in the non-CNS group (p=0.016). Moreover, when the SLE patients were categorized using CNS manifestations indicating neurologic or psychiatric disorders, the anti-APEX nuclease 1 antibody levels were significantly elevated in SLE patients with psychiatric disorders (p=0.037). In conclusion, the association of SLE with several new and previously reported autoantibodies has been demonstrated. Statistically significant associations between anti-esterase D antibodies and CNS syndromes as well as between anti-APEX nuclease 1 antibodies and psychiatric disorders in SLE were also demonstrated. The combined immunoproteomic approaches used in this study are reliable and effective methods for identifying SLE autoantigens.
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INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease that usually develops in women aged 18–50 years and is characterized by the presence of autoantibodies. Diagnosis is difficult because SLE is a great imitator of other diseases (1). Autoantibodies are clearly central to the pathogenesis of SLE, and different autoantibodies are associated with different clinical features (2). Several of the autoantibodies identified to date, numbering over 100, have been associated with disease activity (1). Although anti-double-stranded DNA antibodies are the most extensively studied autoantibodies in SLE, others play roles in clinical manifestations, particularly in autoimmune hemolytic anemia, thrombocytopenia, skin disease, and neonatal lupus (3).

Central nervous system (CNS) lupus is a serious and potentially life-threatening manifestation of SLE, occurring in 37-95% of cases, and is associated with increased risk of death (4). Despite its frequency and severity, the lack of a diagnostic gold standard makes it challenging to differentiate primary CNS lupus from secondary neuropsychiatric (NP) manifestations unrelated to SLE at their onset (4-6). The American College of Rheumatology (ACR) has developed a standardized nomenclature system to provide case definitions for 19 NP syndromes associated with SLE, including reporting standards and recommendations for laboratory and imaging tests (5). Although this standardized nomenclature has helped to clarify a complicated situation, its usefulness as a clinical diagnostic criterion remains to be determined. Significant numbers of reports have found an association between the NP manifestations of SLE and the presence of autoantibodies, although in some cases contrasting data have been reported. The pathogenic role of most of these autoantibodies has not been extensively studied, and they may be merely an epiphenomenon (7). The identification and characterization of new, specific autoantibodies could help elucidate the etiology of the NP manifestations that accompany SLE, opening new perspectives for more effective diagnostic and therapeutic strategies.

Conventionally, study of the autoimmune response has been conducted by analyzing the presence and/or concentration of individual antibodies in biological fluids. Proteomic techniques allow the simultaneous identification and measurement of different autoantibodies in the sera of patients suffering from autoimmune diseases (8). Recent advances in proteomic technologies have enabled large-scale profiling of proteins in tissues and sera from patients and provided an unprecedented ability to identify novel biosignatures useful in diagnosing and classifying autoimmune diseases and guiding therapeutic decision making in patients with these disorders, including SLE (9-15). The possibility of simultaneously measuring a number of correlated analytes is interesting for analytical reasons (e.g., reduced biological sample and reagent volumes and lower costs), logistical and managerial reasons, and pathophysiological reasons (i.e., identifying combinations of markers for use in disease-oriented or organ-oriented profiling) (8).
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However, much work remains unfinished in developing, refining, validating and applying proteomics technologies to identify biomarkers in autoimmune diseases (9). By using both conventional and newer proteomic approaches, our aim was to find novel serum autoantibodies associated with SLE, focusing on those found in patients with CNS syndromes.

EXPERIMENTAL PROCEDURES

Study subjects and sample collection

Sera from 106 patients with active SLE from 1994 through 2007 were obtained using the Tokyo Women’s Medical University SLE Database. These sera were originally collected from the patients’ whole blood using standard tubes with a polyester gel separator. Immediately after clotting at room temperature and following centrifugation, the separated sera were aliquoted and stored at –80°C. All patients had 4 or more revised ACR (formerly the American Rheumatism Association) criteria for SLE (16, 17) and gave informed consent for inclusion in this study. Those who had non-SLE-related NP manifestations arising from infection, uremia, electrolyte imbalance, hypoxia, brain tumor, trauma, primary mental disease, drug use or past histories of NP involvement were excluded. These patients were excluded because we wanted to compare recently diagnosed, active CNS lupus patients to non-NPSLE patients; unrelated conditions could affect current symptoms or laboratory findings. At the time of serum collection, each patient completed a standardized medical history that included medication use and was given a physical examination that included neurologic and rheumatologic assessments. Psychiatric examinations were employed when indicated. Serology profiling for each patient was performed using standard immunoassays. Subjects were classified into the CNS group or the non-CNS group according to the presence or absence of active CNS syndromes. The CNS group was then further classified into the neurologic disorders group consisting of patients with neurologic disorders with or without other NP syndromes, or the psychiatric disorders group comprising patients with psychiatric disorders with or without other NP syndromes (5, 18, 19). Detailed diagnostic criteria for these groups are described below. Control sera were derived from age- and sex-matched healthy donor subjects and from patients with rheumatoid arthritis (RA), systemic sclerosis (SSc), Sjögren syndrome (SS), and multiple sclerosis (MS) diagnosed using standard criteria (20-23). This study was approved by the Ethical Committee of our institution and the Helsinki Declaration was followed throughout the study.

Diagnosis of CNS lupus

Although ACR nomenclature and case definitions include 12 CNS syndromes and 7 peripheral nervous system syndromes (5, 18, 19), we utilized only the 12 CNS syndromes in the
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Inclusion criteria for our study because of the substantial differences between the central and peripheral nervous systems in anatomy, function, and clinical characteristics. Slight or mild cognitive dysfunction without significant clinical impairment, as revealed by detailed neuropsychological testing, was excluded from the CNS syndromes in our study. Tension headache and episodic tension type headache were also excluded. CNS syndromes were further classified into neurologic disorders (aseptic meningitis, cerebrovascular disease, demyelinating syndrome, headache, movement disorder, myelopathy, seizure disorders) and psychiatric disorders (acute confusional state, anxiety disorder, cognitive dysfunction, mood disorder, psychosis) (5).

The final clinical diagnosis and classification of the various NP syndromes for inclusion in the study were made by an experienced rheumatologist (M. H.) and psychiatrist (K. N.), according to the standardized ACR nomenclature and case definitions for NP lupus syndromes (5). These decisions were based on the medical history and neuropsychological examinations by rheumatologists, an experienced neurologist (S. U.) and a psychiatrist (K. N.) and were supported by conventional laboratory tests and appropriate complementary tests, including MRI, electroencephalography, and cerebral spinal fluid tests, as well as an assessment of the clinical course of the disease.

Cell culture

Human neuroblastoma cell lines (IMR-32 and NB-1) and human glioblastoma cell lines (A172 and T98G) were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). IMR-32 cells were routinely cultured in MEM (Sigma-Aldrich, St. Louis, MO) supplemented with non-essential amino acids (MP Biomedicals, Irvine, CA). NB-1 cells were cultured in a medium containing an equal amount of MEM and RPMI-1640 (Sigma-Aldrich). The human glioblastoma cell lines, A172 and T98G, were cultured in RPMI-1640. All culture media were supplemented with 10% FBS (SAFC Biosciences, Lenexa, KS), 50 U/ml of penicillin, and 50 µg/ml of streptomycin.

Protein preparation for screening of candidate autoantigens

The total protein from human whole brain (BioChain Institute, Hayward, CA) was precipitated once using the ReadyPrep 2-D Cleanup Kit (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer’s protocol and used for 2D polyacrylamide gel electrophoresis. The cultured cells were washed with PBS, scraped into a 1.5 ml tube and centrifuged to harvest cells. To prepare total cell protein, the cell pellet was sonicated on ice [output: 2; duty: 60; for 1 minute (min); Sonifier 250D; Branson Ultrasonics Corporation, Danbury, CT] in 6 M urea, 2 M
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thiourea, 4% CHAPS, and a complete EDTA-free protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). After centrifugation at 20,400 x g for 10-30 min at 4°C, the supernatant was recovered as total cell protein for use in the screening of autoantigens. Similarly, as for total protein from human whole brain, the total cell protein was precipitated using the ReadyPrep 2-D Cleanup Kit. Protein concentrations were measured using the bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL) according to the manufacturer’s protocol, using BSA as the standard.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Human brain proteins were screened for autoantigens using 2 proteomic analysis techniques. First, we performed 2D-PAGE/western blots (WBs) and then analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF/MS). 2D-PAGE was performed as described elsewhere (24). Briefly, prepared protein samples were precipitated using the ReadyPrep 2-D Cleanup Kit (Bio-Rad) according to the manufacturer’s protocol. Before electrophoresis, the protein pellets were dissolved in DeStreak Rehydration Solution (GE Healthcare, Buckinghamshire, UK) containing 0.2% BioLyte 3/10 (Bio-Rad) and 50 mM dithiothreitol (DTT). Next, 50 µg of protein was applied to IPG ReadyStrip (pH 3-10, non-linear, 7 cm long; Bio-Rad) for overnight in-gel rehydration. The proteins were then separated by 1D isoelectric focusing (1D-IEF) using Multiphore II (GE Healthcare) or CoolPhoreSter IPG-IEF Type-P (Anatech, Tokyo, Japan) at 20°C and focused with the following program: 200 V for 18 min, 500 V for 18 min, 1000 V for 18 min, 1500 V for 18 min, 2000 V for 18 min, 2500 V for 18 min, 3000 V for 18 min, and 3500 V for 90 min. Following 1D-IEF, the IPG strips were equilibrated in a solution of 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 0.002% bromophenol blue that also contained 10 mg/ml DTT for the first equilibration step and 25 mg/ml iodoacetamide in the second equilibration step; the strips were bathed in solution on an orbital shaker at room temperature for 15 min in each step. The equilibrated strips were then sealed with 1% SeaKem GTG Agarose (Lonza, Rockland, ME) in SDS running buffer at the top of 2D SDS-polyacrylamide gels. SDS-PAGE was performed using precast 5-20% polyacrylamide gradient gels (SuperSep 5-20%, 2D gel; Wako Pure Chemical Industries, Osaka, Japan) at a constant current of 20 mA/gel until the bromophenol blue dye front reached the lower edge of the gel.

WB analysis of 2D-PAGE using patient sera

After 2D-PAGE, the separated proteins were transferred onto Immobilon-P (Millipore, Billerica, MA) with a semidry blotter (ATTO AE-6677; ATTO, Tokyo, Japan) at a constant current of 2 mA/cm² for 1 hour (h) with a solution of 100 mM Tris base, 192 mM glycine, and
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5% methanol. Membranes were washed with 0.1% Tween 20 in PBS (PBS-T). Before WB, proteins on the membrane were labeled using Cy5 Mono-Reactive Dye (GE Healthcare) for 30 min in the dark at room temperature; any remaining free dye was removed by washing with PBS-T. The membranes were blocked for 1 h at room temperature with blocking buffer (1% skim milk in PBS-T). WBs were performed using the pooled sera from SLE patients with active CNS syndromes (the CNS group) or those without either current CNS syndromes or a history of CNS syndromes (the non-CNS group) diluted 1:500 in blocking buffer and incubated overnight at 4°C. After being washed with PBS-T, the membranes were incubated for 1 h at room temperature with the secondary antibody goat anti-human IgG+A+M (H+L) (Zymed, San Francisco, CA), conjugated to horseradish peroxidase (HRP), and diluted 1:15,000 in blocking buffer. WBs were visualized using the ECL plus (GE Healthcare) chemifluorescence signal, and proteins on the membrane were visualized using the Cy5 fluorescence signal. These fluorescent images were scanned using a Typhoon 9400 (GE Healthcare) with excitation at 457 nm and emission filter 520BP40 (ECL plus) and with excitation at 633 nm and emission filter 670BP30 (Cy5). The resulting images were overlaid and easily matched to identical positions from the western signal and protein signal. Using this information, protein samples were prepared for mass spectrometry analysis.

MALDI-TOF/MS
Using the results of the 2D-PAGE/WB, the ECL plus signal spots specific for CNS lupus patients' pooled sera were detected, and their positions were identified on the Cy5-protein signals. For MALDI TOF/MS analysis, the proteins were separated using 2D-PAGE, transferred onto a ProBlott membrane (Applied Biosystems, Foster City, CA) and stained with Coomassie Brilliant Blue (CBB) R-250 (PhastGel Blue R, GE Healthcare). CNS lupus-specific spots were excised and washed with Milli-Q water. The isolated proteins were then digested at 37°C for 90 min using 1 pmol/µl of lysine endopeptidase (mass spectrometry grade, Wako Pure Chemical Industries) in 50% acetonitrile. The resulting peptides were purified using NuTip NT1HIL.96 solid-phase extraction cartridges (Glygen, Columbia, MD) and mixed with α-cyano-4-hydroxycinnamic acid (CHCA) matrix. Peptide mass fingerprinting was performed using a MALDI-TOF mass spectrometer (Voyeger™, Applied Biosystems). Peptide mass fingerprinting data were compared to the NCBI Inr databases (human, 233173 sequences; date 2010/12/24) using the Mascot Search engine (Peptide Mass Fingerprint, version 2.2; Matrix Science, Boston, MA) with the following parameters: Enzyme, Lys-C; Variable modifications, Carbamidomethyl (C) and Oxidation (M); Mass values, Monoisotopic; Protein Mass, Unrestricted; Peptide Mass Tolerance: ±0.2-0.4 Da; Peptide Charge State, 1+; and Max Missed Cleavages, 0 and exceeded the thresholds \( p < 0.05 \). Confidence in the reliability of the
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**Preparation of antigens for immunoprecipitation**

Because 2D-PAGE/WB and MALDI TOF/MS analyses did not have sufficient sensitivity, we also utilized immunoprecipitation and protein shotgun analyses using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to screen candidate autoantigens. In the first trial, 4 types of cell lines (NB-1, IMR-32, A152 and T98G) were cultured, washed with PBS, and harvested. Cell pellets were suspended in a cell lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM MgCl₂, 10 mM NaF, 2 mM EGTA, 25 mM beta-glycerophosphate, 2 mM DTT, 1% NP-40, 10% glycerol, and 0.2% benzonase nuclease [Novagen, Darmstadt, Germany]) containing a protease inhibitor cocktail (Roche, Basel, Switzerland) and incubated on ice for 30 min. After centrifugation at 20,400 × g for 10 min at 4°C, the supernatants were recovered and protein concentrations were measured using the BCA protein assay. Then, 5 mg of cell lysate for each of the 4 cell lines was mixed. In a separate assay, 10 mg of total protein from human whole brain (BioChain Institute) was added to this mixture of lysates from the 4 types of cell lines (5 mg each). These antigen mixtures were precleared by incubation with 150 µl Protein G Sepharose beads (GE Healthcare) to prevent nonspecific proteins from binding to the beads and then used as antigens for immunoprecipitation.

**Preparation of total IgG immobilized by Protein G Sepharose beads**

Total IgG from 67.5 µl of serum mixtures from 9 SLE patients with active CNS syndromes and from 9 SLE patients without active CNS syndromes was incubated with 37.5 µl of Protein G-Sepharose beads at room temperature using a gentle rocking motion for 45 min. The beads were then centrifuged and the supernatant was removed. The beads were washed twice with 0.2 M borate buffer (pH 9.0) and the IgG was crosslinked to the Protein G Sepharose beads by incubation at room temperature with 5 mM disuccinimidyl suberate (DSS, Pierce) for 30 min. The beads were washed with 0.2 M glycine (pH 8.0) and again incubated at room temperature for 30 min to block the active sites of DSS. The beads were then equilibrated with cell lysis buffer.

**Immunoprecipitation of autoantigens and total IgG**

A total of 7.5 mg of the antigens prepared as described above was added to the IgG immobilized beads and incubated at 4°C for 90 min with agitation. After centrifugation, the pellets were washed with washing buffer (20 mM HEPES, pH 7.5, 650 mM NaCl, 2 mM MgCl₂, 10 mM NaF, 2 mM EGTA, 25 mM beta-glycerophosphate, 1% NP-40, and 10% glycerol)
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**Sample Preparation for LC-MS/MS Analysis**

The samples were prepared for LC-MS/MS analyses as previously described (28). Briefly, the autoantigen pellet was dissolved in 100 μl of 1.2 M urea and 80 mM Tris-HCl, pH 8.5. The autoantigen was digested with trypsin (250 ng added directly to the solution at 37 °C for 16 h, Trypsin Sequencing Grade, modified; Roche), desalted using a ZipTip (C18; Millipore), and finally concentrated to approximately as 20 μl to inject into the nanoflow system.

**Protein shotgun analysis by LC-MS/MS**

Protein shotgun analyses were performed using LC-MS/MS as previously described (28). Briefly, after the peptide mixture was applied to a C18 column (800-μm-inner-diameter and 3-mm-long), reversed-phase separation of the captured peptides was performed using a column (150-μm-inner-diameter and 75-mm-long) filled with HiQ-Sil C18 (3-μm particles, 120-Å pores; KYA Technologies, Tokyo, Japan) with a direct nanoflow LC system (Dina, KYA Technologies). The peptides were eluted with a linear 5–65% gradient of acetonitrile containing 0.1% formic acid over 120 min at a flow rate of 200 nl/min and sprayed into a quadrupole time-of-flight tandem mass spectrometer (Q-Tof™ 2, Micromass/Waters, Milford, MA) (29). The MS/MS spectra were acquired using the following parameters: dynamic exclusion time, 120 s; duty cycle, 2 s; and mass tolerance, 0.1 Da. The MS/MS signals were then converted to text files by MassLynx (version 3.5, Micromass/Waters) and processed against the NCBI RefSeq human protein database (38963 sequences as of July 5, 2010) using the Mascot algorithm (MS/MS Ion Search, version 2.2.04, Matrix Science) with the following parameters: variable modifications, Acetyl (Protein N-term), Gln->pyro-Glu (N-term Q), and Oxidation (M); maximum missed cleavages, 2; peptide mass tolerance, 200 ppm; and MS/MS tolerance, 0.5 Da. The criterion for protein identification was based on having at least one MS/MS data with Mascot scores that exceeded the thresholds ($p < 0.05$). A randomized decoy database created by a Mascot Perl program estimated a false discovery rate at 0.48% for all of the identified peptides.

**Production of recombinant proteins of identified antigens**

Based on the screening results of the 2 proteomic approaches described above and also literature information, possible antigens were selected. The cDNAs of those candidate autoantigens were amplified by RT-PCR from the total RNA extracted from human brain or NB-1 neuroblastoma cells using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA).
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These cDNAs were then cloned into the expression vector pQE-Tri System (QIAGEN, Alameda, CA), pET-52b(+) (Novagen) or pQE30 (QIAGEN). The recombinant His-tag fusion proteins were expressed in *E. coli* and purified with Ni-NTA agarose (QIAGEN) and/or TALON metal affinity resin (Clontech, Mountain View, CA). The purity of each recombinant protein was checked by SDS-PAGE, and its reactivity against autoantibodies was assessed by WB using sera from SLE patients and controls.

**Serum IgG autoantibodies reactivities against the recombinant antigens by WB**

The reactivities of serum IgG autoantibodies against the recombinant antigens in sera from the original SLE patients were examined by WB, as described below. Recombinant antigens were separated using SDS-PAGE on a precast gel (NPG-520L, ATTO) and blotted onto an Immobilon-P membrane (Millipore) using a semidry blotter (ATTO). The membranes were blocked for 1 h at room temperature with 1% skim milk in PBS-T blocking buffer. The membranes were then cut into strips, and WBs were performed using patient or control serum (diluted 1:500 in blocking buffer) and anti His-tag monoclonal antibodies (QIAGEN, diluted 1:5000) overnight at 4°C. After being washed with PBS-T, the membranes were reacted for 1 h at room temperature with the secondary antibody, HRP-conjugated sheep anti-human IgG, diluted 1:15,000 in blocking buffer. The membranes were then incubated with the substrate (Western Lightning Plus; PerkinElmer, Waltham, MA) and visualized by exposure to Kodak X-OMAT Blue XB film (Kodak, Tokyo, Japan).

**Quantification of autoantibody reactivities against the major discriminant antigens in the sera of patients and controls by enzyme-linked immunosorbent assay (ELISA)**

Finally, autoantibodies found in the sera of patients with SLE, patients with other autoimmune diseases, and normal healthy controls (NHCs) were quantified by employing solid phase direct ELISAs using the positive recombinant human brain antigens as previously described (30), with slight modifications. Purified autoantigen (0.1-5.0 µg/ml in PBS) was coated onto the wells of a microtiter plate (Nunc, Roskilde, Denmark) by incubation overnight at 4°C. The wells were then blocked for 2 h using 200 µl of PBS containing 1% BSA at room temperature. After the plate was washed 5 times with 400 µl of PBS containing 0.1% Tween 20, 100 µl of diluted serum sample (diluted 1:200 in PBS-T containing 0.3% BSA) was incubated in each well at room temperature for 2 h. After the plate was washed, 100 µl of diluted HRP-conjugated anti human IgG (diluted 1:20,000 in PBS-T containing 0.3% BSA) was added to each well and the plate was incubated at room temperature for 1 h. After the plate was washed, 100 µl of substrate solution (0.04% o-phenylenediamine dihydrochloride with 0.012% H₂O₂) was added to each well, and the plate was incubated at room temperature for 30 min. The
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reaction was stopped by the addition of 100 µl of 2 N H$_2$SO$_4$. The absorbance was measured at 492 nm using a microplate reader. Titers for the autoantibodies were expressed as the mean O.D. values of the triplicate wells. The numbers of samples examined varied among the ELISA antigens because of sample and antigen availability.

Statistical analyses

The results of the ELISAs were correlated with the final clinical diagnosis for each patient. Two-group comparisons were analyzed using the Mann–Whitney $U$ test for continuous variables. The Steel multiple comparison test was applied when appropriate. Values of $p < 0.05$ were considered statistically significant. Sensitivity, specificity, positive predictive value, and negative predictive value for the diagnosis were also calculated for some ELISAs. All statistical analyses were performed using JMP statistical software (version 7.0; SAS Institute, Cary, NC).

RESULTS

Clinical characteristics of SLE patients

Of the 106 patients with SLE enrolled in the present study, 100 were women and 6 were men. The median age of the patients was 31 years (ranging from 16 to 68 years). The median disease duration since the diagnosis of SLE was 1 year (with a range of 0 to 20 years). The patients were all Japanese, except for 1 woman who was Chinese. Recently (i.e., within the last month) diagnosed, active CNS syndromes were observed in 32 patients (the CNS group), while the remaining 74 patients had neither current CNS syndromes nor a history of CNS syndromes (the non-CNS group). A neurologic disorder was diagnosed in 21 subjects, a psychiatric disorder was diagnosed in 15 subjects, and both disorders were diagnosed in 4 subjects. The final clinical diagnoses and classifications of the various NP syndromes for inclusion in the study were made according to the standardized ACR nomenclature and case definitions for NP lupus syndromes (5, 18, 19). There were no significant differences in other clinical parameters such as sex, age, or disease duration between the CNS group and the non-CNS group ($p = 0.78, 0.78, \text{ and } 0.68$, respectively).

Autoantigen screening by 2D-PAGE and WB

Autoantigens were screened for this study using 2 methods of proteomic analysis. First, we performed 2D-PAGE/WB and MALDI TOF/MS analysis, a rather conventional approach for proteomic analysis (9). Total protein obtained from human whole brain or cell lysates of human neuroblastoma cell lines IMR-32 and NB-1 or human glioblastoma cell lines A172 and T98G was prepared as described in the Experimental Procedures and separated using 2D-PAGE. After
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2D-PAGE, the separated proteins were transferred onto membranes. Before WB, the proteins on the membrane were labeled with Cy5 Mono-Reactive Dye to allow the determination of their positions. The WB procedure was performed using pooled sera from SLE patients with active CNS syndromes (the CNS group) or those without either current CNS syndromes or a history of CNS syndromes (the non-CNS group), and proteins were visualized using ECL plus chemifluorescence. The resulting images were overlaid and easily matched to identical positions based on the western signal and protein-conjugated dye signal (Fig. 1, A and B). Using this information, protein samples were prepared for mass spectrometry.

Identification of proteins by MALDI-TOF/MS and Mascot Search (Peptide Mass Fingerprint)

Differentially reacted protein spots specific to the CNS group were excised from the blotted membranes and subjected to digestion with lysine endopeptidase, followed by peptide-mass fingerprinting and MALDI-TOF/MS. The peptide mass fingerprints obtained were used to search the NCBInr database using the Mascot Search engine. Twelve protein spots from whole human brain total protein and 4 from human tumor cell line total protein were significantly and exclusively detected with pooled sera from the CNS group (circled in Fig. 1, A and B, respectively, and summarized in Table I). Although many spots were found by this method, most of them were difficult to identify because of their low expression levels; too many minor proteins were present in each spot for the sensitivity of this method.

Autoantigen screening by immunoprecipitation of human brain proteins with SLE patient total IgG

2D-PAGE is labor intensive and often has the disadvantages of poor reproducibility, lack of sensitivity and low throughput. Several newer approaches have recently been introduced to address some of these limitations in quantitative serum proteome analysis (9). Because 2D-PAGE/WB followed by MALDI-TOF/MS (2D-PAGE/WB/MALDI-TOF/MS) analysis did not have sufficient sensitivity for our study, we explored a new approach utilizing immunoprecipitation followed by LC-MS/MS shotgun analysis. Total protein from human whole brain and human brain tumor cell lines was prepared as described above in the Experimental Procedures. Total IgG from serum mixtures from SLE patients with active CNS syndromes (the CNS group) or those without either current CNS syndromes or a history of CNS syndromes (the non-CNS group) was bound to Protein G-Sepharose beads. Prepared antigens were immunoprecipitated using the patients' total IgG immobilized onto Protein G Sepharose beads.
Identification of the proteins by LC-MS/MS shotgun analysis and Mascot Search (MS/MS Ion Search)

The autoantigen protein pellets were digested using trypsin, and the peptide fragments were analyzed on a nanoflow LC-MS/MS system. The LC-MS/MS shotgun analyses were performed twice for each sample group: the CNS and the non-CNS SLE groups. Proteins were identified from the MS/MS spectra and the NCBInr database using the Mascot Search engine (MS/MS Ion Search). A total of 154 proteins, most not found using the 2D-PAGE/WB/MALDI-TOF/MS analysis, were identified using this method (as shown in the supplemental data). Among these proteins, 50 were identified in the CNS group, but not in the non-CNS group (summarized in Table II).

WB analysis of serum IgG autoantibodies reactivities against discriminant recombinant antigens

Although some of the proteomic data (e.g. esterase D) were insufficient for protein identification by themselves (supplemental data), we selected possible antigens based on not only the results of the 2 proteomic approaches described above but also literature information. Among the many candidate antigens screened, 11 recombinant antigens showed positive reactions against the pooled sera of 28 patients with active CNS lupus by WB analysis: crystallin αB, esterase D, APEX nuclease 1, 60S acidic ribosomal protein P0 (ribosomal protein P0), proteasome activator complex subunit 3 (PA28γ), triosephosphate isomerase, phosphoglycerate kinase 1 (cell migration-inducing gene 10 protein), phosphatidylethanolamine-binding protein 1, phosphoglycerate mutase 1, 3-hydroxyacyl-CoA dehydrogenase type-2, and glial fibrillary acidic protein (Fig. 2). Among these, APEX nuclease 1, PA28γ, phosphoglycerate mutase 1, 3-hydroxyacyl-CoA dehydrogenase type-2, and glial fibrillary acidic protein were identified exclusively by immunoprecipitation followed by LC-MS/MS shotgun analysis. Established SLE-associated autoantibodies, such as anti-SS-A/Ro antibodies, were excluded from the present study, although they were detected in our proteomic analyses.

Autoantibodies in the sera of patients with SLE and other autoimmune diseases and NHCs reactive against the major discriminant antigens quantified by ELISA

We validated these 11 autoantigens using sera from SLE patients with or without active CNS syndromes. Serum samples from 106 SLE patients, including 42 patients with active CNS syndromes, and from 100 NHCs were subjected to ELISA to quantify the autoantibodies reactive against these antigens. The original sera of SLE patients used in the screening and verification by WB were included in these final samples. The O.D. values of SLE patient serum samples were significantly higher than those of NHCs in ELISAs using the following 5 antigens: crystallin αB (p = 0.0002), esterase D (p = 0.0002), APEX nuclease 1 (p < 0.0001), ribosomal protein P0 (p <
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0.0001), and PA28γ ($p = 0.0005$) (Fig. 3). To our knowledge, the association of SLE and autoantibodies against crystallin αB, esterase D, or APEX nuclease 1 has not been previously reported. To assess the disease specificity for SLE of these autoantibodies, we also performed ELISAs of crystallin αB or APEX nuclease 1 using sera from patients with RA, SSc, SS, and MS; esterase D was not studied due to the limited volumes of antigen available. The ELISA-generated O.D. values for sera from SLE patients were significantly higher than those for patients with the other diseases and NHCs for APEX nuclease 1 (SLE vs. all other controls using the Steel multiple comparison test; $p < 0.0001$), but not significantly different for crystallin αB (see Fig. 4, A and B, respectively). Using a theoretical cutoff titer of 0.39 (O.D.) for anti-APEX nuclease 1 antibodies as the NHC mean+3SD, their prevalence in patients with SLE, RA, SSc, SS, and MS and NHCs was 20%, 13%, 8%, 5%, 5%, and 2%, respectively. When samples of SLE patients and NHCs were compared, the sensitivity, specificity, positive predictive value, and negative predictive value of the anti-APEX nuclease 1 antibodies for the diagnosis of SLE were 20%, 98%, 95%, and 33%, respectively.

Comparisons of the SLE-related autoantibody titers among the CNS group and the non-CNS group using ELISA

The O.D. values of sera from the CNS group were significantly higher than those from the non-CNS group in ELISAs using esterase D ($p = 0.016$), although most of them show low O.D. values (< 0.1; Fig. 5A). Unexpectedly, no O.D. values in ELISAs of other autoantibodies differed significantly between SLE patients with or without active CNS syndromes when tested in larger sample sizes: crystallin αB ($p = 0.75$), APEX nuclease 1 ($p = 0.18$), ribosomal protein P0 ($p = 0.89$), or PA28γ ($p = 0.58$). However, when the CNS group with neurologic disorders and the CNS group with psychiatric disorders were separately compared with SLE patients without those disorders, the levels of anti-APEX nuclease 1 antibodies were significantly elevated in SLE patients with psychiatric disorders compared with the levels in SLE patients without psychiatric disorders ($p = 0.037$; Fig. 5B).

**DISCUSSION**

The major findings of this study were the following: (i) associations between SLE and several new and previously reported autoantigens/autoantibodies were demonstrated using multiple proteomic analyses: 2D-PAGE/WB/MALDI-TOF/MS analysis and immunoprecipitation followed by LC-MS/MS shotgun analysis; and (ii) statistically significant associations between anti-esterase D antibodies and CNS syndromes as well as between anti-APEX nuclease 1 antibodies and psychiatric disorders in SLE were also shown by ELISAs,
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although the associations of other autoantibodies with CNS syndromes were unexpectedly unclear.

We found new, previously unreported associations of 3 serum autoantibodies with SLE, demonstrating reactivity against crystallin αB, esterase D, and APEX nuclease 1. Crystallin αB is a small stress protein with cytoprotective and anti-apoptotic functions that is abundant in the eye lens, but its constitutive expression at high levels is otherwise restricted to a limited number of tissues with high oxidative function, such as myocardial and skeletal muscle (31). Humoral responses against crystallin αB have been observed in patients with inflammatory nervous system diseases and Guillain-Barré syndrome, implying it has a role in the pathophysiology of these disorders. However, we could find no previous report of a specific association between crystallin αB and SLE in the literature. Human esterase D (carboxylesterase) is one member of the nonspecific esterase family defined by their reaction with synthetic o-acyl ester substrates (32). It has been found in most tissues, but the highest levels of this enzyme are found in the liver and the kidney. It has been suggested that esterase D may have a role in detoxification. Autoantibodies against esterase D produced in experimental autoimmune uveoretinitis mice were also detected in some endogenous uveitis human patients (33). However, we also found no previous reports of a specific association between esterase D and SLE or CNS syndromes. APEX nuclease 1 (also known as multifunctional DNA repair enzyme 1) is a mammalian major apurinic/apyrimidinic endonuclease that plays a central role in DNA base excision repair (34). It is also a redox factor, stimulating the DNA-binding activity of several transcription factors. APEX is expressed throughout development in all tissues and its expression is sensitive to changes in cellular conditions including oxidative stress, wound healing, and hypoxia. In the present study, anti-APEX nuclease 1 antibodies were specifically detected in the sera of SLE patients, but not in other diseases or in NHCs. Again, we found no previous reports in the literature of an association between APEX nuclease 1 and SLE or CNS syndromes.

Our proteomic analyses also identified 2 autoantibodies with previously reported associations with SLE: ribosomal protein P0 and proteasome activator complex subunit 3 (PA28γ/Ki). Several other established SLE-associated autoantigens, such as Sm and SS-A/Ro, were also identified by our proteomic screening. These results attest to the reliability of the immunoproteomic approach used in this study for identifying autoantigens in SLE. Phosphorylated ribosomal (P ribosomal) proteins are 3 ubiquitous, highly conserved acidic phosphoproteins (P0, P1, and P2) that play roles in protein synthesis (35). The ribosomal protein P0 localizes to the membrane surface of neuronal, hepatic, and endothelial cells in an immunologically accessible way. Antibodies to the P ribosomal proteins are considered highly specific markers for SLE and appear to correlate with disease activity for the liver and kidney disease as well as with CNS involvement. Proteasomes were originally described as cytoplasmic,
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ATP-dependent, proteolytic enzyme complexes involved in antigen presentation on MHC class I molecules. More recent structural analyses, however, show the nuclear antigen Ki (PA28γ) to be an element of proteasomes. Moreover, studies examining the biological function of the proteasome show that it also catalyzes proteolysis for metabolism and protein quality control, apoptosis, signal transduction, and cell-cycle regulation. Recent advances in immunohistology have revealed that some neurodegenerative diseases, including Alzheimer’s disease and Parkinson’s disease, may result from a dysfunction of the ubiquitin-proteasome system. Antibodies to Ki (PA28γ) have been reported in some patients with SLE.

Statistically significant associations between anti-esterase D antibodies and CNS syndromes as well as between anti-APEX nuclease 1 antibodies and psychiatric disorders in SLE were also demonstrated for the first time. However, although promising, these novel findings require cautious interpretation and further investigation to allow for decisive conclusions because most of the O.D. values of anti-esterase D antibodies in the present study were not very high and there were several samples from patients without psychiatric disorders that had high-titer anti-APEX1 antibodies and vice versa. In addition, the biological relationship between these ubiquitous proteins and CNS-specificity was not clear in the present study. In the assessment of other antibodies, although WB analyses with smaller sample sizes showed significant specificity, most of the ELISA results with larger sample sizes indicated that they had insufficient specificity to be useful biomarkers for CNS lupus. The reason for these conflicting results is unclear, but may be related to the heterogeneity of patients with CNS lupus or methodological differences between WB and ELISA. It is worth mentioning that various forms of CNS lupus syndromes were pooled, which could confound the results of the present study. In addition, the ubiquitous proteins examined in the present study are probably less specific for CNS syndrome-related autoantigens. More promising results may be obtained by focusing on specific antigens localized exclusively in the brain.

In addition to ours, a spectrum of potential new biomarkers in SLE has been detected by 2D-PAGE/WB/MALDI-TOF/MS analyses. For example, a few brain antigenic targets were characterized in NPSLE patients using MALDI-TOF/MS analysis (10). However, only very small numbers of samples were analyzed in these studies, and larger validation studies are needed to establish the clinical relevance of these markers and their suitability for clinical assay development (9). Moreover, there are disadvantages of 2D-PAGE/WB/MALDI-TOF/MS analysis including poor reproducibility, lack of sensitivity, low throughput and a considerable workload (9). Several approaches to address the limitations of 2D-PAGE/WB/MALDI-TOF/MS analysis have recently been introduced (9). However, immunoprecipitation followed by LC-MS/MS shotgun analysis used in the present study has rarely been applied for systemic autoimmune diseases such as SLE so far.
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Although many protein spots were found by 2D-PAGE/WB/MALDI-TOF/MS analysis in our study, most were difficult to identify for several reasons. First, too many minor proteins in each spot could not be identified due to the limited sensitivity of the method. Second, the sera of active SLE patients usually contain many high-titer, high-affinity polyclonal antibodies (3). Finally, these antibodies often recognized minor proteins compared to cancer-related proteins. In contrast, more than 150 proteins were identified using immunoprecipitation followed by LC-MS/MS shotgun analysis. Thus, when samples that include many high-titer polyclonal antibodies, such as those found in the sera of active SLE patients, are tested for autoantigens, the immunoproteomic approach used in this study combining 2D-PAGE/WB/MALDI-TOF/MS analysis and immunoprecipitation followed by LC-MS/MS shotgun analysis is an effective and reliable method.

In conclusion, the association of SLE with several new and previously reported autoantibodies was demonstrated using 2 proteomic approaches. Among them, statistically significant associations between anti-esterase D antibodies and CNS syndromes as well as between anti-APEX nuclease 1 antibodies and psychiatric disorders in SLE were also shown. This study demonstrates that the immunoproteomic approach used in this study combining 2D-PAGE/WB/MALDI-TOF/MS analysis and immunoprecipitation followed by LC-MS/MS shotgun analysis is a reliable and effective method for the identification of autoantigens in SLE. Determining the precise roles of newly identified SLE-related autoantibodies in the pathogenesis of SLE and their usefulness as biomarkers will require further study.

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REFERENCES


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Figure Legends

**Figure 1.** 2D-PAGE/WB of total human brain protein using sera from CNS lupus patients. Human brain proteins were screened for autoantigens by 2D-PAGE followed by WB and MALDI TOF/MS analysis. (A) WB following 2D-PAGE against total protein of human whole brain using sera from CNS lupus patients. (B) WB following 2D-PAGE against total protein of human brain tumor cell lines using sera from CNS lupus patients. The identified differentially expressed protein spots are summarized in Table I.

**Figure 2.** Representative results of WB analysis using serum IgG autoantibodies against discriminant recombinant human brain antigens. Among the many candidate antigens identified by 2D-PAGE followed by WB and MALDI TOF/MS analysis as well as by immunoprecipitation followed by LC-MS/MS shotgun analysis, there were 11 recombinant antigens showing positive reactions against pooled sera from 28 active CNS lupus patients by WB. Representative results are shown. The membranes were cut into strips. Pooled sera of 15 non-CNS SLE patients and those of 8 NHCs were used as controls. Anti-His indicates the result of anti-His-Tag antibodies that recognize His-Tags placed at N-terminal, C-terminal, and internal regions of fusion recombinant proteins. (A) Crystallin \( \alpha \B); (B) Esterase D; (C) APEX nuclease 1; (D) 60S acidic ribosomal protein P0 (ribosomal protein P0); (E) Proteasome activator complex subunit 3 (PA28\( \gamma \)).

**Figure 3.** Representative results of ELISAs using sera from SLE patients against recombinant autoantigens. Serum IgG autoantibodies against the 11 selected recombinant autoantigens were validated by ELISA using serum samples from 106 SLE patients, including 42 patients with active CNS syndromes, and 100 NHCs. Positive results are shown for (A) Crystallin \( \alpha \B, (B) Esterase D, (C) APEX nuclease 1, (D) 60S acidic ribosomal protein P0 (ribosomal protein P0), and (E) Proteasome activator complex subunit 3 (PA28\( \gamma \)). Titers for the autoantibodies are expressed as the mean O.D. values of the triplicate wells. \( P \) values were calculated using the Mann–Whitney \( U \) test.

**Figure 4.** Autoantibodies against selected antigens in the sera of patients with SLE and other autoimmune diseases as assessed by ELISA. The disease specificities of selected autoantibodies were assessed by ELISA using sera from patients with RA, SSc, SS, or MS. Titers for the autoantibodies were expressed as the mean O.D. values of the triplicate wells. The ELISA O.D. values for the sera of SLE patients were significantly higher than those of patients with other diseases or the NHC for (A) APEX nuclease...
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1 (SLE vs. all the other controls by the Steel multiple comparison test; \( p < 0.0001 \)), but did not
differ significantly for (B) Crystallin \( \alpha B \). The horizontal dotted line in Figure 4A indicates a
theoretical cutoff titer of 0.39 (O.D.) for anti-APEX nuclease 1 antibodies determined as the
NHC mean+3SD, as described in the Results section.

Figure 5. Associations between autoantibodies against esterase D and APEX nuclease 1 and
CNS syndromes in SLE by ELISAs.
Serum IgG autoantibodies against esterase D were compared between SLE patients with and
without active CNS syndromes by ELISA. Serum IgG autoantibodies against APEX nuclease 1
were compared between SLE patients with and without active psychiatric disorders by ELISA.
Statistically significant associations between anti-esterase D antibodies and CNS syndromes (A)
as well as between anti-APEX nuclease 1 antibodies and psychiatric disorders (B) in SLE are
shown. \( P \) values were calculated using the Mann–Whitney \( U \) test.
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Table I. Identified CNS lupus-specific autoantigens by MALDI-TOF/MS from the 2D-PAGE/western blot of human whole brain and human brain tumor cell line total protein.

CNS = central nervous system; MALDI-TOF/MS = matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; 2D-PAGE = 2-dimensional polyacrylamide gel electrophoresis
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* These numbers show in which 2 LC-MS/MS shotgun analyses the proteins were identified.

* Protein identification was based on the criterion of having at least one MS/MS data with Mascot scores that exceeded the thresholds (p < 0.05).

CNS = central nervous system; LC-MS/MS = liquid chromatography-tandem mass spectrometry.
Fig. 1
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Fig. 2
Fig. 4.

A

Anti-APEX Nuclease1 Ab (O.D.)

SLE (n = 106) RA (n = 40) SSc (n = 20) SS (n = 20) MS (n = 20) NHC (n = 42)

B

Anti-Crystallin Ab (O.D.)

SLE (n = 106) RA (n = 40) SSc (n = 20) SS (n = 20) MS (n = 20) NHC (n = 38)
Fig. 5

A

Anti-Esterase D Ab (O.D.)

CNS-SLE  Non-CNS-SLE

p = 0.016

B

Anti-APEX nuclease 1 Ab (O.D.)

Psychiatric-SLE  Non-Psychiatric-SLE

p = 0.037