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Human Brain Protein Antigens Reacting with Autoantibodies from Systemic Lupus Erythematosus Patients with Central Nervous System Manifestation

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Autoantibodies to neuronal cell antigen have been proposed to be responsible for central nervous system (CNS) disease in patients with systemic lupus erythematosus (SLE). However, the target antigen molecules in human brain tissue responsible for the development of CNS dysfunction in SLE patients have not yet been identified. To identify the specific human brain proteins associated with CNS dysfunction in SLE, we measured the autoantibodies to both human brain proteins and extractable nuclear antigen from calf thymus by ELISA, and characterized the human brain autoantigen by immunoblot analysis using sera from 15 SLE patients with diffuse CNS manifestation, 14 SLE patients without CNS manifestation, and 16 healthy subjects as controls. Although the levels of autoantibodies to both human brain proteins and extractable nuclear antigen were higher in SLE patients than the controls ($p<0.05$), there were no significant differences in the levels between SLE patients with or without CNS manifestation ($p>0.05$). On immunoblot analysis, autoantibodies to 38 kDa protein in human brain tissue were positive in 6 (86%) of 7 SLE patients with CNS manifestation, 2 (29%) of 7 SLE patients without CNS manifestation, and one (20%) of 5 controls, and a significant difference in positive rates was noted between two SLE groups (Chi-square test; $p<0.05$). In conclusion, the 38 kDa human brain protein may be an important target for autoantibodies in sera of SLE patients with CNS manifestation, and further study is essential to confirm the pathogenic significance of this autoantigen. (Ajou Med J 1998; 3(2): 106~111)

Key Words: *Systemic lupus erythematosus, Central nervous system, Autoantibodies, Autoantigen, Enzyme- linked immunosorbent assay, Immunoblot analysis*

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by a presence of a variety of circulating autoantibodies. Clinical manifestations associated with central nervous system (CNS) involvement occur in approximately half of all SLE patients.[1] Organic brain syndrome, psychosis, and seizures are the most common CNS manifestations in SLE patients.[2] However, the pathogenesis of CNS involvement in SLE has not yet been clarified.

Autoantibodies to brain antigen have been proposed to be responsible for central nervous system (CNS) disease in patients with SLE.[3] Indirect immunofluorescence study demonstrated that autoantibodies against brain tissue in sera from SLE patients were mainly localized in the neuronal cells.[4,5] Antineuronal antibodies are frequently detected in sera from patients with SLE, and there is some evidence that the titers of those antibodies vary with lupus activity in the nervous system.[4,5] The association between antineuronal antibodies and CNS manifestation is particularly strong in patients with organic brain syn-

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drome, psychosis, or seizures.[3] Antineuronal antibodies are detected in cerebrospinal fluid[6] and in neuronal tissues of some SLE patients who succumb to CNS involvement.[7] Additional evidence for a pathogenic role of antineuronal antibodies is derived from animal studies in which seizures and neuropathologic changes have been demonstrated following the intrathecal or intravenous infusion of antibodies reactive with nervous tissue antigens.[8]

Most studies on the antineuronal antibodies have used either cultured neuroblastoma cell[9] or animal brain tissue[10] for detection and characterization of antigenic targets of these autoantibodies. However, the human brain target antigen molecule responsible for the development of CNS dysfunction in SLE patient has not yet been identified.[11]

To identify the specific human brain protein antigen associated with CNS dysfunction in SLE, we measured the autoantibodies to both human brain protein and extractable nuclear antigen from calf thymus by enzyme-linked immunosorbent assay (ELISA), and characterized the human brain autoantigen by immunoblot analysis using sera of SLE patients with or without CNS manifestation.

MATERIALS AND METHODS

Serum samples

Peripheral venous blood samples were collected from 15 SLE patients with diffuse CNS manifestation (generalized seizure, organic brain syndrome, severe persistent headache), 14 SLE patients without CNS manifestation who attend out patient clinic and inpatient at Ajou University Hospital, and 16 healthy subjects as controls. Following centrifugation, all sera were aliquoted and stored at $-20^{\circ}C$.

Human brain tissue

Fresh brain and muscle tissues were obtained from grossly normal fetus who underwent autopsy due to abortion. The tissues were stored at $-70^{\circ}C$. Brain and muscle tissues were incubated with an equal volume of lysis buffer containing 40 mM Tris/HCl, pH 8.0, 120 mM NaCl, 0.5% NP-40, 2 μg/ml aprotinine, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride. They were thoroughly homogenized in a glass homogenizer at $4^{\circ}C$. The homogenate was shaken for 2 hr at $4^{\circ}C$ to solubilize membrane fractions and centrifuged at 10,000 g for 20 min to remove debris. The supernatant was frozen in aliquots at $-20^{\circ}C$. The protein content was measured using Bradford's method.[12]

Extractable nuclear antigen

This antigen was obtained from a calf thymus acetone extract (Sigma Chemical Co., USA) as previously described.[13] The lyophilized powder was suspended in phosphate buffered saline at a concentration of 50 mg/ml. It was mixed with vortex mixer for 30 min and centrifuged at 10,000 g for 20 min. The supernatant was frozen in aliquots at $-20^{\circ}C$. The protein content was measured using Bradford's method.[12]

Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (Immulon 2; Dynatech Laboratories, Inc., Chantilly, Va) were coated with 50 μl of human brain protein or extractable nuclear antigen at a concentration of 10 μg/ml in 0.1M carbonate buffer, pH 9.6, overnight at $4^{\circ}C$. Plates were washed with phosphate buffered saline with 0.05% Tween-20 (PBST) and blocked by 3% bovine serum albumin in PBST and then plates were incubated with 50 μl of duplicate serum samples at 1 : 500 dilution in blocking buffer for 2 hrs at room temperature. After washing, plates were incubated with peroxidase-conjugated goat anti-human IgG (Sigma Chemical Co., St. Louis, MO) at 1 : 5000 dilution for 2 hrs at room temperature. After washing, the substrate solution, consisting of 0.04% (w/v) orthophenylenediamine dissolved in 24.3 mM citric acid, 51.4 mM NaH_2PO_4 (pH 5.0) and 0.03% H_2O_2 was added. After 30 min, the reaction was stopped by adding 2.5 N H_2SO_4. Absorbance at 490 nm was measured by a microplate reader (Molecular Devices Corporation, Menlo Park, CA). The levels of specific antibodies were expressed as absorbance values. When the absorbance of sample was higher than mean ± 2 standard deviations of absorbance values from 16 controls, it was regarded as positive for autoantibodies.

ELISA inhibition

ELISA inhibition was performed using a pooled serum sample from three SLE patients. The pooled serum sample diluted at 1 : 500 (v/v) was incubated overnight at 4°C with different concentrations of human brain and muscle proteins or extractable nuclear antigen. They were tested for residual specific IgG antibodies to human brain protein by ELISA as described above.

Immunoblot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described previously[14] with 12% gel. The 9.0×8.0 cm mini-gel (Novex, San Diego, CA) was used, and the sample gel was divided into two lanes by the use of a special comb; one 0.35 cm wide for prestained molecular weight marker standards (Novex, San Diego, CA) and another 8.0 cm wide for a sample application. Human brain homogenate was mixed with sample buffer containing 50 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulphate and 10% glycerol, and adjusted to 1 mg protein/ml concentration and heated in boiling water for 5 min. The gel was loaded with 100 μg of human brain proteins and electrophoresed under unreduced condition. After electrophoresis, the proteins on the gel were electroblotted onto a polyvinylidine difluoride membrane (Novex, San Diego, CA). Then, the membrane was blocked in 10 mM Tris, 0.15 M NaCl, and 5% bovine serum albumin (pH 7.4) for 2 hrs. Membrane strips were incubated with serum samples from SLE patients and healthy controls at a 1 : 100 dilution for 3 hrs at room temperature. After three washes, the membranes were incubated with alkaline phosphatase- conjugated goat anti-human IgG (Sigma Chemical Co., St. Louis, MO) at 1 : 2000 dilution for 2 hrs at room temperature. Finally, after washes, the membranes were incubated with the substrate solution (nitro blue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate; Sigma Chemical Co., St. Louis, MO). The reaction was stopped after 10 min by rinsing the membrane in distilled water.

Statistics

Data were expressed as mean±standard deviation (SD). The Mann-Whitney *U* test was used to assess the differences of specific antibody levels between the two groups. Chi-square test was used to assess the differences of positive rates between the two groups.

RESULTS

Autoantibodies to Human Brain Proteins and Extractable Nuclear Antigen

The levels of autoantibodies to human brain proteins were significantly higher in SLE patients (mean absorbance±SD; 0.602±0.375) than in controls (0.245±0.046) ($p<0.05$, Fig. 1). The levels of autoantibodies to extractable nuclear antigen were significantly higher in SLE patients (0.732±0.515) than in controls (0.207±0.070) ($p<0.05$, Fig. 2). However, the levels of these autoantibodies were not significantly different between SLE patients with or without CNS manifestation ($p>0.05$; Fig. 1, Fig. 2). Autoantibodies to human brain proteins were positive in 80% of SLE patients with CNS manifestation and 64% of SLE patients without CNS manifestation ($p>0.05$). Autoantibodies to extractable nuclear antigen were positive in 53% of SLE with CNS manifestation and 79% of SLE patients without CNS manifestation ($p>0.05$).

ELISA inhibition

Autoantibodies to human brain proteins were inhibited

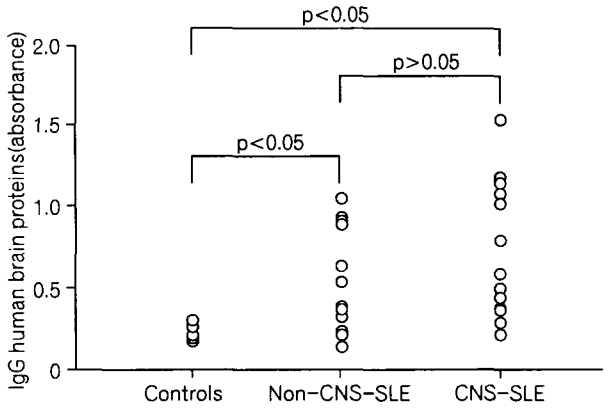

Fig. 1. IgG autoantibodies to human brain proteins in sera from SLE patients without CNS manifestation (Non-CNS-SLE), SLE patients with CNS manifestation (CNS-SLE) and healthy controls.

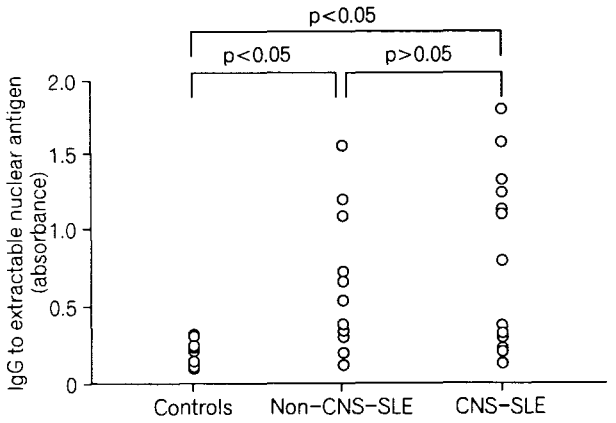

Fig. 2. IgG autoantibodies to extractable nuclear antigen in sera from SLE patients without CNS manifestation (Non-CNS-SLE), SLE patients with CNS manifestation (CNS-SLE) and healthy controls.

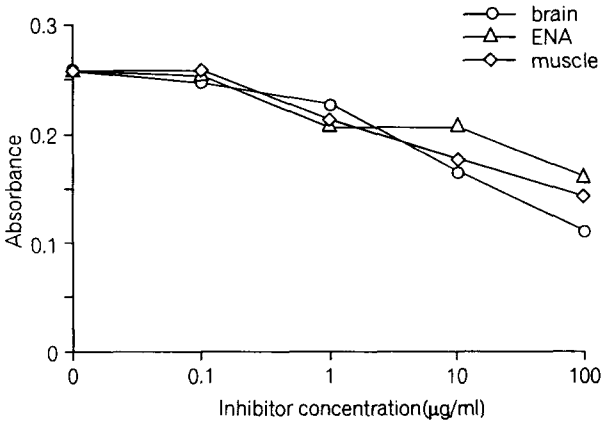

Fig. 3. ELISA inhibition of IgG autoantibodies against human brain proteins in sera from SLE patients by human brain proteins, extractable nuclear antigen (ENA) from calf thymus extract, and human muscle proteins.

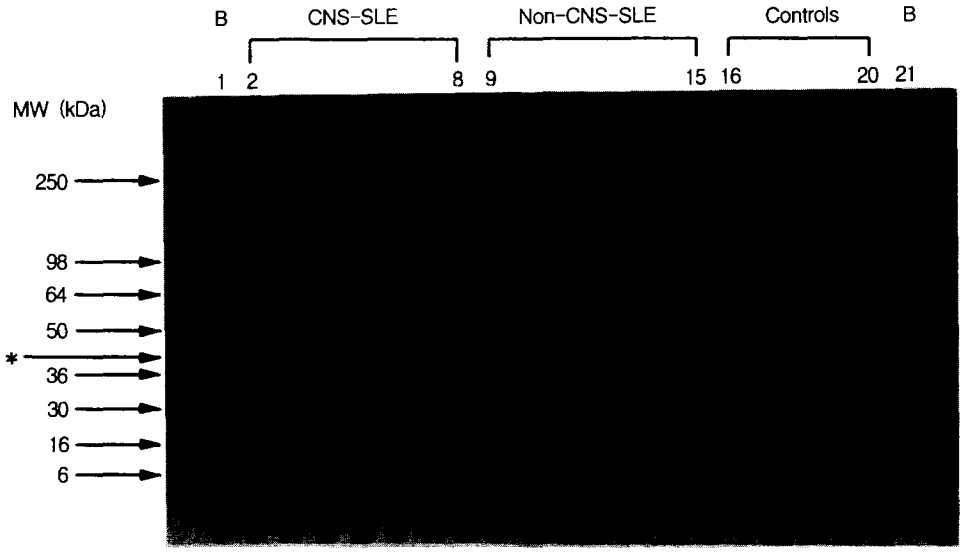

Fig. 4. Immunoblot analysis of IgG autoantibodies to human brain proteins in sera from SLE patients without CNS manifestation (Non-CNS-SLE, lane 2~8), SLE patients with CNS manifestation (CNS-SLE, lane 9~15) and controls (lane 16~20). Lane 1 and lane 21 were negative controls incubated with buffer only (B). An arrow (*) indicates the 38 kDa protein band.

dose-dependently by not only the human brain proteins, but also human muscle proteins and extractable nuclear antigen from calf thymus extract (Fig. 3).

Immunoblot analysis

Autoantibodies in sera from SLE patients recognized at least 15 human brain proteins with molecular weights of 125, 112.5, 92, 72.5, 68, 65, 57.8, 50, 38, 36, 32, 25, 18, 11, and 8 kDa (Fig. 4, Table 1). There were two non-specific binding protein bands between 250 kDa and 98 kDa which were visible in the strip incubated with buffer only. They were regarded to be aggregated heavy and light chains of IgG molecules present in human brain tissue. Autoantibodies to 38 kDa protein in human brain tissue were positive in 6 (86%) of 7 SLE patients with CNS manifestation, 2 (29%) of 7 SLE patients without CNS manifestation, and one (20%) of 5 controls, and a

Table 1. *Summary of immunoblot analysis data of IgG autoantibodies to human brain proteins in sera from SLE patients without CNS involvement (Non-CNS-SLE), SLE patients with CNS involvement (CNS-SLE), and healthy controls*

Sample No.	CNS-SLE							Non-CNS-SLE							Controls				
MW(kDa)	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
125	+	+	+	+	+	+	+	+	+		+			+	+		+	+	
112.5	+	+	+	+	+		+			+	+		+	+					
92										+									
72.5	+					+													
68	+						+												
65												+							
57.8	+						+		+						+				
50	+				+	+	+	+	+		+			+	+				
38	+	+	+	+	+		+				+		+						+
36	+						+					+							
32	+				+		+				+	+	+						+
25							+												
18							+												
11	+						+				+								

significant difference in positive rates was noted between the two SLE groups (Chi-square test; $p<0.05$).

DISCUSSION

The present study showed that autoantibodies to 38 kDa human brain protein might be associated with CNS manifestation in SLE patients. There are several autoantigen molecules found to be associated with CNS manifestations of SLE patients.[15] One study[10] found an autoantibody reactivity with a 50 kDa neuronal antigen associated with active CNS lupus in 19 of 20 patients tested, and an another study[9] found a 97 kDa antigen present on neuronal cells associated with the presence of clinical CNS manifestation of SLE patients. However, these studies used neuroblastoma cell line or animal brain tissue as the antigen source. Although technically less difficult, they may detect antigens associated with tumor or species specific antigen to those animals. To avoid these problems, the use of human brain tissue as an antigen source was recommended.[11]

An association of antibodies against ribosomal P proteins with psychosis and /or depression in SLE patients has received a wide attention,[16] although this remains to be controversial.[15] Of great interest is the fact that some form of ribosomal P proteins (P0 protein) could be expressed on the surface of neuroblastoma cells and, therefore potentially could become the target of autoantibodies in vivo.[17] Interestingly, this protein has a molecular weight of 38 kDa,[17] which is identical with autoantigen shown in this study.

Although we defined the clinical manifestation of SLE patients according to the medical records retrospectively, there were no clinical manifestations to suggest psychosis such as irrational speech or hallucinations in SLE patients included in this study. However, we cannot rule out completely a possibility of subclincal symptoms or a presence of unrecorded psychotic events in these patients. Other unclear points in this study is the fact that the 38 kDa molecule in human brain is not well characterized; Whether this molecule is intracellular or membrane-bound or whether this molecule is specific to brain tissue or not. Furthermore, the tested sample size in this study is too small in order to prove definitely an association of the autoantibodies with CNS manifestation of SLE patients. Characterization of this protein by molecular biological technique and further studies with a larger sample size is in need.

The diagnosis of CNS involvement in SLE is complicated, because CNS manifestations can also occur as a secondary phenomenon caused by other manifestations of the diseases such as uremia, hypertension, infections or even by steroid treatment.[18] Routine serological tests such

as complement or anti-DNA antibody levels are less reliable indicators for active CNS involvement in SLE.[18] Therefore, a useful serologic tool for diagnosis is needed. A test for autoantibodies to the neuroblastoma cell line has been proposed for this purpose, however, its diagnostic value is controversial.[18] In this study, we observed no significant difference in the levels of autoantibodies to human brain proteins of SLE patients with CNS manifestation and those without. ELISA inhibition study suggests that a major portion of autoantibodies to human brain proteins are not specific to brain tissue, but rather might be directed to intracellular nuclear antigens. An approach to find a clinically relevant antigen and to use recombinant antigen seems to be necessary for serologic diagnosis of CNS involvement in SLE patients.

In conclusion, 38 kDa human brain protein may be an important target for autoantibodies in sera from patients with CNS manifestation, and further study is essential to confirm the pathogenic significance of this autoantigen.

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