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PAPER

Flow cytometric assessment of anti-neuronal antibodies in central nervous system involvement of systemic lupus erythematosus and other autoimmune diseases

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The objective of this study is to evaluate the association between anti-neuronal antibody (anti-NA) and central nervous system (CNS) manifestations of systemic lupus erythematosus (SLE) and other rheumatic diseases using a flow cytometric method. Anti-NA was measured by flow cytometry in serum and cerebrospinal fluid (CSF) samples from patients with SLE ($n = 44$ for serum, $n = 17$ for CSF), other rheumatic diseases ($n = 64$ for serum, $n = 21$ for CSF) and from healthy controls ($n = 65$ for serum, $n = 18$ for CSF). Serum anti-NA was more frequently observed in SLE (31.8%, 14/44) than in other rheumatic diseases (4.7%, 3/64, $P < 0.001$) or in healthy controls (0%, 0/65, $P < 0.00001$). In SLE patients, the frequency of serum anti-NA was significantly higher in CNS-SLE (76.5%, 13/17) than in non CNS-SLE (3.7%, 1/27, $P < 0.000001$). CSF anti-NA was detected in 88.2% (15/17) of CNS-SLE and was more frequently detected in CNS-SLE (15/17, 88.2%) than in other rheumatic diseases with CNS involvement (1/21, 4.8%, $P < 0.000001$) or in healthy controls (0/18, $P < 0.000001$). In conclusion, serum anti-NA was more frequently found in CNS-SLE than in non CNS-SLE, other rheumatic diseases or in healthy controls. The frequency of CSF anti-NA in CNS-SLE was significantly higher than in other rheumatic diseases with CNS involvement or in healthy controls. *Lupus* (2008) 17, 21–25.

Key words: anti-neuronal antibody; central nervous system; lupus erythematosus; systemic

Introduction

Neuropsychiatric involvement occurs in up to two-thirds of systemic lupus erythematosus (SLE) patients, and these patients exhibit a wide spectrum of clinical manifestations that can be grouped as neurologic or psychiatric.¹ The pathogenesis of central nervous system (CNS) involvement in SLE (CNS-SLE) is not well understood and is believed to involve more than one mechanism. Accordingly, it is difficult to diagnose CNS-SLE and currently, diagnoses are arrived at clinically based on the exclusion of other possible etiologies. Although computed tomography and magnetic resonance imaging (MRI) are used as complementary

tests, they often produce nonspecific results and do not reflect pathophysiological changes.

It has been reported that anti-neuronal antibody (anti-NA) in serum, and particularly in cerebrospinal fluid (CSF), is associated with CNS-SLE.^{2–5} However, reported prevalences of anti-NA in SLE vary widely^{3,6} and anti-NA has also been observed in SLE with no clinical evidence of CNS involvement.⁷ The diversity of results regarding anti-NA in CNS-SLE appears in part to be due to the different assay techniques used and the patients included.^{3–6,8,9} Of the several methods developed to determine anti-NA, recent studies in SLE patients have tended to use solid phase immunoassays like cell-ELISA.^{4,5} The disadvantage of these methods is that they involve the use of fixed cells rather than intact cells, and therefore, there is a risk of cellular entry by autoantibodies and their reaction with intracellular antigens. Here, we assayed anti-NA with flow cytometry using an unfixed neuroblastoma cell line (SK-N-MC) and investigated the association between anti-NA and the CNS involvement in SLE patients and in patients with other rheumatic diseases.

[†]Both authors contributed equally to this study.

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Materials and methods

Patients and samples

Forty-four SLE patients who had been admitted to Seoul National University Hospital from November 1999 to December 2005 were enrolled in the study. Nineteen of them were identified to have CNS symptoms and/or signs. All patients fulfilled four or more of the 1997 American College of Rheumatology (ACR) revised criteria for SLE.¹⁰ Patients were diagnosed as having CNS-SLE, if they had significant neurological or psychiatric dysfunctions identified by history, physical examination and laboratory and radio logic tests and by subsequent clinical course and response to treatment, as required by the ACR criteria for CNS involvement of SLE.¹ Serum and CSF samples were obtained from SLE patients ($n = 44$ for serum, $n = 19$ for CSF), from patients with other rheumatic diseases ($n = 64$ for serum, $n = 21$ for CSF), which included primary Sjögren's syndrome (SS), rheumatoid arthritis (RA), multiple sclerosis (MS), systemic sclerosis (SSc), neuro-Behçet's disease (neuro-BD) and primary angiitis of the CNS, and from healthy controls (Table 1). All serum and CSF samples were collected when CNS signs and/or symptoms were present and were stored at -80°C in a refrigerator until required for assay. This research was approved by the Institutional Review Board of Seoul National University Hospital.

Measurement of anti-NA

Anti-neuronal antibody in serum and CSF samples was detected by flow cytometry using SK-N-MC cells (a human neuroblastoma cell line).¹¹ Briefly, cells were subcultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. After detaching cells from culture vessels using 0.125% trypsin in 0.02% EDTA, they were washed and gently re-suspended in phosphate-buffered saline (PBS) at 2×10^6 cells/mL. After

blocking nonspecific binding sites with 10% fetal calf serum, 100 μL of cell-containing PBS was incubated with 10 μL of serum or 100 μL of CSF for 30 min at room temperature. Unbound antibodies were removed by washing twice with PBS. Cells were then stained with phycoerythrin-conjugated goat anti-human IgG (Rockland Immunochemicals, Inc., Gilbertsville, PA, USA) and fixed in 1% formaldehyde (Sigma, St Louis, MO, USA). Analysis was performed using a flow cytometer (BD Biosciences, San Jose, CA, USA). Upon running the first tube (assay calibrator sample), the PE photomultiplier tube voltage was adjusted so that the median channel fluorescence (MCF) value of the histogram profile was within the target channel (<1.0). A total of 2000 gated SK-N-MC cell events were collected for analysis. Serum or CSF was considered positive when the MCF value was greater than the means plus three standard deviations (SD) of normal sera ($n = 65$) or normal CSF ($n = 18$).

Measurement of other autoantibodies

Titers of anti-Sm, anti-Ro, anti La and anti-ribosomal P antibody in sera ($n = 44$) and CSF ($n = 19$) of SLE patients were measured by enzyme-linked immunoassay at RDL Reference Laboratories (Los Angeles, CA, USA). Serum anti-double-stranded DNA (anti-dsDNA) titer was measured by radioimmunoassay (Johnson & Johnson Clinical Diagnostics, Rochester, NY, USA).

Review of medical records

Medical charts of 19 SLE patients with CNS symptoms and/or signs were reviewed with respect to demographic background, disease duration, specific symptoms and laboratory and radiological data including MRI findings.

Statistical analysis

Mean values were compared using the Mann-Whitney U -test. The χ^2 -test was used for categorical comparisons, and the Spearman's rank test was used to assess correlations. The Statistical Package for the Social Sciences (SPSS), version 11.0 (SPSS, Chicago, IL, USA), was used for the analyses. P -values of <0.05 were considered significant.

Results

Among the 19 SLE patients who exhibited CNS symptoms, two had other etiologies for their CNS symptoms (e.g., carbon dioxide narcosis due to interstitial lung

Table 1 Profiles of patients enrolled in this study

Diagnosis	Number of patients (with CNS involvement)
Systemic lupus erythematosus	44 (17)
Other rheumatic diseases	64 (21)
Sjogren's syndrome	20 (2)
Rheumatoid arthritis	15 (0)
Multiple sclerosis	13 (13)
Systemic sclerosis	10 (0)
Neuro-Behçet's disease	5 (5)
Primary angiitis of central nervous system	1 (1)
Healthy control	65 (18)

disease; seizure due to sequela of a previous cerebral infarct) and consequently 17 patients were diagnosed as having CNS-SLE. The mean age (\pm SD) of CNS-SLE patients and the percentage of women in these patients were 37.6 ± 19.7 years and 88.2% (15/17), respectively. Of the 64 patients with other rheumatic diseases, 21 had CNS involvement (2 SS, 13 MS, 5 BD, 1 primary angiitis of CNS).

Anti-neuronal antibody in the sera of patients with SLE or other rheumatic diseases

The cut-off MCF value used to define a positive serum anti-NA assay was 1.25. The MCF values of SLE ($n = 44$) and of other rheumatic diseases ($n = 64$) are shown in [Figure 1A]. Serum anti-NA was more frequently observed in SLE (31.8%, 14/44) than in other rheumatic diseases (4.7%, 3/64, $P < 0.001$) or in healthy controls (0%, 0/65, $P < 0.000001$). Thirteen CNS-SLE patients (76.5%, 13/17) were positive for serum anti-NA whereas only one nonCNS-SLE patient (3.7%, 1/27) was positive ($P < 0.000001$). The sensitivity and specificity of serum anti-NA for CNS involvement in SLE were 76.5 and 96.3%, respectively and the positive and negative likelihood ratios were 20.7 and 0.014, respectively. All three (2 RA, 1 SS) patients with other rheumatic diseases positive for serum anti-NA were free from CNS involvement.

Anti-neuronal antibody in the CSF of patients with SLE or other rheumatic diseases

Cerebrospinal fluid samples from CNS-SLE patients ($n = 17$) and patients with other rheumatic diseases with CNS involvement ($n = 21$) were analysed [Figure 1B]. The cut-off MCF value used to define a positive CSF anti-NA assay was 1.01. Cerebrospinal fluid anti-NA assays were positive in 15 of 17 CNS-SLE patients (88.2%, 15/17), whereas only one sample from patients with other rheumatic diseases (MS) was positive (4.8%, 1/21, $P < 0.000001$). None of the CSF samples from healthy controls showed anti-NA (0%, 0/18, $P < 0.000001$). All CNS-SLE patients with serum anti-NA also showed CSF anti-NA. Serum and CSF anti-NA were found to be well correlated ($r = 0.581$, $P = 0.014$) (Figure 2).

Clinical features and anti-NA status in CNS-SLE

As shown in Table 2, the clinical syndromes of the 17 CNS-SLE patients included intractable headache ($n = 7$), seizure disorder ($n = 4$), acute confusional state ($n = 3$), aseptic meningitis ($n = 1$), cerebrovascular disease ($n = 1$) and psychosis ($n = 1$). Among these 17 patients, CSF anti-NA was detected in 15

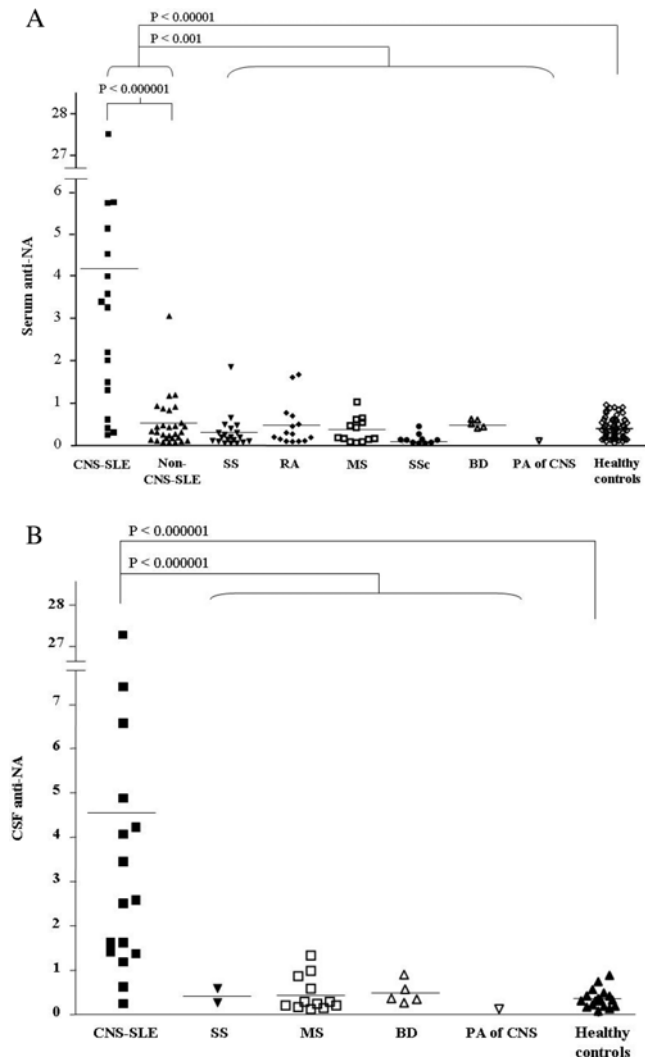


Figure 1 Anti-neuronal antibody antibody reactive to the surface antigen of the SK-N-MC cells (a human neuroblastoma cell line) was detected by flow cytometry. Median channel fluorescence (MCF) values of serum (A) or cerebrospinal fluid (CSF) samples (B) are shown for individuals. The bars indicate mean MCF values. CNS, central nervous system; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; RA, rheumatoid arthritis; MS, multiple sclerosis; SSc, systemic sclerosis; BD, Behçet's disease; PA, primary angiitis.

(88.2%); intractable headache (6/7, 85.7%), seizure disorder (4/4, 100%), acute confusional state (3/3, 100%), aseptic meningitis (1/1, 100%) and cerebrovascular disease (1/1, 100%). On the other hand, MRI abnormalities were found in 10 patients (58.8%); intractable headache (3/7, 42.9%), seizure disorder (4/4, 100%), acute confusional state (2/3, 66.7%) and cerebrovascular disease (1/1, 100%) (Table 2). The MRI abnormalities observed in CNS-SLE varied from multiple high signal intensities in cortical, subcortical and/or periventricular areas, cerebral edema, to diffuse

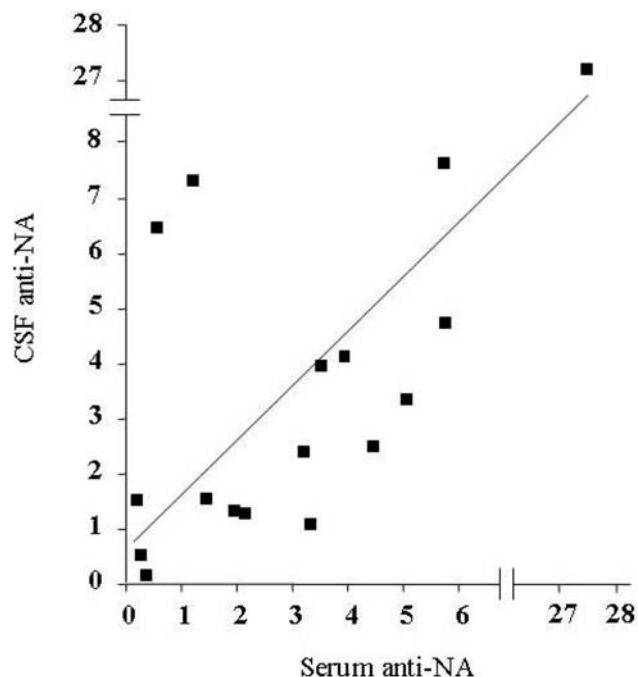


Figure 2 Correlation between serum and cerebrospinal fluid (CSF) anti-NA antibody levels in SLE patients with CNS involvement. A good correlation ($r = 0.581$, $P = 0.014$) was found between the MCF values of serum and CSF anti-NA antibodies.

Table 2 Central nervous system manifestations and anti-NA in CNS-SLE patients ($n = 17$)

CNS manifestations (Number)	Number of anti-NA positivity (%)		Number of MRI abnormality (%)
	CSF	serum	
Intractable headache (7)	6 (85.7)	6 (85.7)	3 (42.9)
Seizure disorder (4)	4 (100)	3 (75)	4 (100)
Acute confusional state (3)	3 (100)	3 (100)	2 (66.7)
Aseptic meningitis (1)	1 (100)	1 (100)	0 (0)
Cerebrovascular disease (1)	1 (100)	0 (0)	1 (100)
Psychosis (1)	0 (0)	0 (0)	0 (0)
Total (17)	15 (88.2)	13 (76.5)	10 (58.8)

leptomeningeal enhancement and did not show any specific pattern for a given syndrome.

Other autoantibodies and their associations with anti-NA in CNS-SLE

Anti-ribosomal P antibody was detected in four sera (three headaches and one acute confusional state) and three CSF samples (all with headache) of the 17 CNS-SLE patients (23.5, 17.6%, respectively). Serum anti-dsDNA antibody was detected in six CNS-SLE patients (6/17, 35.3%). No significant correlation was found between serum anti-NA and anti-dsDNA antibody titers ($r = 0.431$, $P = 0.084$), and no association was found between anti-NA and anti-Sm, anti-Ro or anti-La antibodies.

Discussion

The prevalence of CNS involvement has been reported to range from 14% to 75% in SLE patients and this CNS involvement is recognized as one of the major causes of mortality.¹² However, the pathogenesis of CNS-SLE remains poorly understood. IgG anti-NA was first reported in CNS-SLE based on radioimmunoassays using SK-N-SH cells (a human neuroblastoma cell line).¹³ Subsequently, a variety of other neuronal substrates, such as, brain or neuron-like cells, were used in combination with different methods for detecting anti-NA, for example, immunofluorescence, cell-ELISA and western blotting.^{3,5,8,9} Although there has been a discrepancy in prevalence of the anti-NA in CNS-SLE detected by these various methods, anti-NA in serum and particularly in CSF have been reported to be associated with diffuse CNS-SLE. In agreement with previous reports, the present study shows that serum anti-NA is significantly more prevalent in CNS-SLE than in nonCNS-SLE patients (76.4 versus 3.7%, $P = 0.00000045$), demonstrating a strong association between serum anti-NA and the CNS involvement of SLE. There have been studies reporting that anti-NA in CSF rather than in serum is associated with CNS-SLE.^{4,5} The present study shows that the frequency of CSF anti-NA tended to be higher than that of serum (88.2% versus 76.5%, $P = 0.12$) in CNS-SLE.

Our results concur with those of Alosachie *et al.*, who also found a significantly higher prevalence of serum anti-NA in CNS-SLE (60%) than in nonCNS-SLE (12%) using a flow cytometric assay.¹¹ The present study also shows that: the anti-NA titers of CSF and serum from CNS-SLE correlate well with each other; the anti-NA of CNS-SLE tends to be more frequently detected in CSF than in serum; and that the presence or the titer of anti-NA is not associated with the presence or titer of anti-dsDNA antibody. The results of our study and those of Alosachie *et al.*¹¹ suggest that flow cytometric assays provide sufficient sensitivity (76.5 and 60%, respectively) and specificity (96.3 and 88%, respectively) to justify their use as a tool for detecting anti-NA. However, it needs to be determined whether anti-NA plays a functional role in the CNS manifestations of SLE.

It is worth noting that anti-NA elevation was more frequently observed in CNS-SLE than an abnormal MRI finding, especially in intractable headache patients, which suggests that anti-NA assays might be helpful to diagnose CNS-SLE in patients with only nonspecific symptoms.

In conclusion, in the present study, serum anti-NA detected by flow cytometry was more frequently observed in SLE than in other rheumatic diseases and was found to be associated with CNS involvement in

SLE patients. The prevalence of CSF anti-NA was found to be higher in CNS-SLE than in other rheumatic diseases with CNS involvement or in healthy controls.

Acknowledgement

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