

Elevation of serum anti-glucose-regulated protein 78 antibodies in neuropsychiatric systemic lupus erythematosus

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ABSTRACT

Objective Recent studies have demonstrated that autoantibodies directed against glucose-regulated protein 78 (GRP78) on endothelial cells promote blood-brain barrier (BBB) damages. The present study examined whether serum anti-GRP78 antibodies might be involved in the pathogenesis of neuropsychiatric SLE (NPSLE).

Methods Serum samples were obtained from 129 patients with SLE (58 patients with diffuse psychiatric/neuropsychological syndromes of NPSLE (diffuse NPSLE), 30 with neurological syndromes (focal NPSLE), 21 with lupus nephritis (LN), 20 without NPSLE or LN (SLE alone)), from 35 patients with non-SLE rheumatic diseases (non-SLE RD) and from 24 healthy controls (HC). Anti-GRP78 levels were measured with an ELISA, using recombinant GRP78 as antigens. Cerebrospinal fluid (CSF) samples were also obtained from 88 patients with NPSLE. The BBB function was evaluated by Q albumin ((CSF albumin/serum albumin) $\times 10^3$).

Results Serum anti-GRP78 levels were significantly elevated in SLE compared with non-SLE RD or HC. There were no significant differences in serum anti-GRP78 levels among NPSLE, LN and SLE alone. Of note, serum anti-GRP78 levels were significantly higher in acute confusional state (ACS) than in non-ACS diffuse NPSLE ($p=0.0001$) or in focal NPSLE ($p=0.0002$). Finally, serum anti-GRP78 levels were significantly correlated with Q albumin ($r=0.294$, $p=0.0054$) in NPSLE.

Conclusion These results indicate that anti-GRP78 antibodies are associated with the development of diffuse NPSLE, especially ACS. Thus, the data suggest that anti-GRP78 antibodies might contribute to the development of ACS through the damages of BBB.

INTRODUCTION

Neuropsychiatric SLE (NPSLE) is one of the recalcitrant complications of the disease, leading to substantial impairment of quality of life as well as disability.¹ Among a variety of manifestations in NPSLE, acute confusional state (ACS) in diffuse psychiatric/neuropsychological syndromes (diffuse NPSLE) is the most serious one, requiring extensive immunosuppressive therapy and sometimes resulting in poor prognosis.^{1,2}

N-Methyl-D-aspartate (NMDA) receptors are one of the glutamate receptor families.³ Recently, it has been disclosed that autoantibodies to NMDA receptor subunit NR2 (anti-NR2) play an important role in the development of brain damages in mouse.⁴ More importantly, it was shown that such neuronal damage requires the influx of anti-NR2 into the central nervous system (CNS) through a breakdown of the blood-brain barrier (BBB).⁵

In human, it was also found that cerebrospinal fluid (CSF) anti-NR2, but not serum anti-NR2, were significantly higher in diffuse NPSLE than in focal NPSLE.⁶ Furthermore, CSF anti-NR2 were shown to be elevated in patients with ACS compared with those in non-ACS diffuse NPSLE or in focal NPSLE.⁷ More importantly, the elevation of CSF anti-NR2 in patients with ACS has been shown to result from the damage of BBB, but not from the increased intrathecal production thereof.⁷ Thus, it has been demonstrated that BBB damages play a crucial role in the pathogenesis of diffuse NPSLE, especially ACS. However, the mechanism of the BBB breakdown in NPSLE remained unclear.

Neuromyelitis optica spectrum disorder (NMOSD) is a severe inflammatory autoimmune disorder of the CNS.⁸ Previous studies discovered that serum IgG from patients with NMOSD contained autoantibodies specific for aquaporin-4 (anti-AQP4), the brain's main water channel protein, primarily expressed on CNS astrocytes.⁹ Thus, detection of anti-AQP4 in sera from patients facilitates clinical diagnosis of NMOSD.¹⁰ However, it remained unclear how anti-AQP4 penetrates BBB to access astrocytes.

Glucose-regulated protein 78 (GRP78) is a stress protein belonging to the Hsp70 multi-gene family.¹¹ Previous studies demonstrated that GRP78 is expressed on the surface of cell



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membrane and is released on activation.¹¹ Moreover, it was found that small amounts of free GRP78 as well as naturally occurring anti-GRP78 autoantibodies were detected in the peripheral blood of normal healthy individuals.¹¹ Of note, it was demonstrated that high titres of autoantibodies to GRP78 were induced in mice immunised with recombinant Ro52.¹² Thus, it was suggested that the association of immunogenic proteins with GRP78 might promote autoimmune responses, including those to GRP78 itself.¹²

Cell surface expression of GRP78 in human lung microvascular endothelial cells or human umbilical vascular endothelial cells has been described, but no consistent relationship to endothelial integrity has been reported.^{13,14} Recently, Shimizu *et al* have demonstrated that GRP78 is localised on the cell surface of brain microvascular endothelial cells.¹⁵ Thus, they have identified antibodies from a panel of monoclonal recombinant antibodies established from CSF plasmablasts of patients with NMOsD that strongly bound to brain microvascular endothelial cells and decreased their expression of claudin5.¹⁵ Unbiased membrane proteomics identified GRP78 as the target of these recombinant autoantibodies.¹⁵ Accordingly, repeated administration of anti-GRP78 caused extravasation of serum albumin, IgG and fibrinogen in mice.¹⁵ Thus, it has been suggested that anti-GRP78 antibodies might facilitate the entry of anti-AQP4 into the CNS, allowing the access of these antibodies to astrocytes and resulting in NMO attacks.¹⁵

The expression of a variety of autoantibodies is a hallmark of SLE.¹⁶ It is thus possible that anti-GRP78 might be expressed in patients with SLE. The current studies were therefore designed in order to examine whether anti-GRP78 might be involved in the development of NPSLE.

PATIENTS AND METHODS

Patients and samples

One hundred and twenty-nine patients with SLE were included in the present study. All patients fulfilled the American College of Rheumatology 1982 revised criteria for the classification of SLE.¹⁷ Of the 129 patients with SLE, 58 showed diffuse NPSLE according to the 1999 American College of Rheumatology definition of NPSLE,¹ whereas 30 patients showed neuropsychiatric manifestations other than diffuse NPSLE, including neurological syndromes and peripheral nervous system involvement (focal NPSLE) (table 1). Twenty-one patients had lupus nephritis (LN). There were no patients with overlapping LN and NPSLE. Twenty patients with SLE had neither NPSLE nor LN (SLE alone). As a control, 24 normal healthy subjects (HC) and 35 patients with non-SLE rheumatic disease (non-SLE RD) (18 patients with rheumatoid arthritis and 17 patients with Behcet's disease) were studied.

Cerebrospinal fluid specimens were obtained from the 88 patients with NPSLE by a lumbar puncture on the same

Table 1 Profiles of patients with neuropsychiatric SLE (NPSLE)

Diagnosis	Number of patients	Gender (male/female)	Age (mean±SD)
NPSLE	88	12/76	38.4±15.6
Diffuse NPSLE	58	7/51	37.1±12.4
Acute confusional state	28		
Anxiety disorder	3		
Cognitive dysfunction	9		
Mood disorder	10		
Psychosis	8		
Focal NPSLE	30	5/25	40.2±12.8
Cerebrovascular disease	8		
Demyelinating syndrome	1		
Headache	3		
Meningitis	1		
Movement disorder	2		
Myelitis	1		
Seizure disorder	12		
Polyneuropathy	2		
Lupus nephritis	21	3/18	39.3±14.8
II	1		
III	3		
IV	6		
V	5		
III+V	3		
IV+V	3		
SLE alone	20	4/16	35.3±16.0
Non-SLE rheumatic disease	35	11/24	37.3±12.6
Rheumatoid arthritis	18	6/12	38.1±11.4
Behcet's disease	17	5/12	36.4±14.8
Healthy control	24	8/16	27.3±5.4

day serum samples were obtained, when the diagnosis of NPSLE was made by neurologists and rheumatologists. These samples were kept frozen at -30°C until they were assayed. All assays were performed without knowledge of the diagnosis or clinical presentations.

All the patients with SLE had been hospitalised in Teikyo University Hospital, Kitasato University Hospital or other correlated Hospitals between 1993 and 2015.

Measurement of anti-GRP78 antibodies

Serum anti-GRP78 were determined by ELISA.¹¹ Wells of a 96-well microtitre plate (Nunc, Roskilde, Denmark) were coated with recombinant GRP78 (2 $\mu\text{g}/\text{mL}$) (Enzo Life Sciences, Farmingdale, New York, USA) in phosphate buffered saline (PBS), pH 7.2, at 4°C overnight.

Each well was then overcoated with 2% bovine serum albumin (BSA; Sigma, St. Louis, Missouri, USA) in PBS at room temperature for 3 hours. Prior to being added to the antigen-coated wells, serum samples were usually diluted 1:100 in PBS containing 1% BSA. After incubation at room temperature for 2 hours, bound antibody was detected with peroxidase-conjugated F(ab')₂ fragments of goat anti-human IgG (Cappel Laboratories, Cochranville, Pennsylvania, USA). After incubation with substrate solution containing 40 mg *o*-phenylenediamine and 10 μ L of 30% H₂O₂ in 100 mL of 0.05 M citrate phosphate buffer (pH 4.8) at 37°C for 30 min, the reaction was stopped by addition of 5N H₂SO₄. The absorbance (optical density) at 492 nm (OD492) was read with a two-wavelength microplate photometer (MTP450; Corona Electric, Ibaraki, Japan). Determinations of OD492 were normalised to standard sera for anti-GRP78 such that anti-GRP78 activity might be converted to an arbitrary unit scale.

The concentration of anti-GRP78 that produced half of the maximal absorbance at 492 nm, given by saturating concentrations of anti-GRP78 in the ELISA plate, was arbitrarily defined as 1 U/mL. Non-specific binding activities of the serum samples to wells without GRP78 were also determined by the same unit scale, in reference to the standard curves for specific binding activities to recombinant GRP78. The specific anti-GRP78 activity of each sample was thus determined by subtracting the values for the non-specific binding activity from those for the specific binding activity.

Measurement of antibodies against neuronal cells

Antibodies against neuronal cells (anti-N) in serum samples were determined by cell ELISA using human neuroblastoma cell line SK-N-MC, as previously described.¹⁸ The intra-assay and inter-assay coefficients of variation for anti-N determination were 6.9% and 7.5%, respectively. Furthermore, the cell ELISA has been shown to detect antibodies to cell surface antigens specifically expressed on SK-N-MC cells.¹⁸

Quantitation of albumin and evaluation of BBB function

Albumin in CSF and sera was measured by ELISA using Human Albumin ELISA Quantitation Set (Bethyl Laboratories, Montgomery, Texas, USA). BBB function was evaluated by Q albumin (CSF albumin \times 1000/serum albumin).^{7,19}

Statistical analysis

Differences in various parameters among various groups of SLE were analysed by Kruskal-Wallis test with Dunn's multiple comparison test. Correlations between various variables were determined by Spearman's rank correlation test. All tests were carried out using GraphPad Prism V.7.03 (GraphPad Software, San Diego, California, USA).

RESULTS

Serum anti-GRP78 levels in various subsets of SLE

Initial experiments examined serum levels of anti-GRP78 in various diseases. The baseline variables in various groups of SLE are shown in table 2. There were no significant differences in the parameters in the four groups of SLE, except for CH50, C3 and C4, which were significantly higher in focal NPSLE compared with diffuse NPSLE.

As shown in figure 1, serum anti-GRP78 levels were significantly elevated in 129 patients with SLE (6.66 \pm 20.46 U/mL (mean \pm SD)) compared with 35 patients with non-SLE RD (2.63 \pm 2.94 U/mL) or 24 HC (2.96 \pm 3.04 U/mL). There were no significant differences in serum anti-GRP78 levels among NPSLE (n=88, 5.74 \pm 19.42 U/mL), LN (n=21, 14.95 \pm 30.53 U/mL) and SLE alone (n=20, 2.05 \pm 2.98 U/mL) (figure 2). Of note, however, serum anti-GRP78 levels were significantly higher in ACS (n=28, 13.07 \pm 25.82 U/mL) than those in non-ACS diffuse NPSLE (anxiety disorder, cognitive dysfunction, mood disorder and psychosis) (n=30, 4.20 \pm 20.63 U/mL) or in focal NPSLE (n=30, 0.43 \pm 1.27 U/mL) (figure 3). The results indicate that serum anti-GRP78 levels are elevated in a fraction of patients with NPSLE and with LN. Moreover, the data have disclosed that serum anti-GRP78 are involved in the development of ACS in diffuse NPSLE.

Table 2 Baseline variables in various groups of SLE

Group	n	WBC (/ μ L)	Lymphocytes (/ μ L)	Anti-DNA antibodies (IU/mL)	CH50 (U/mL)	C3 (mg/dL)	C4 (mg/dL)
Diffuse NPSLE	58	5051 \pm 3107 (n=53)	743 \pm 677 (n=47)	95 \pm 136 (n=52)	22 \pm 15 (n=50)	52 \pm 29 (n=50)	11 \pm 10 (n=50)
Focal NPSLE	30	7351 \pm 4284 (n=23)	840 \pm 491 (n=19)	85 \pm 215 (n=25)	*37 \pm 18 (n=23)	*82 \pm 31 (n=22)	*17 \pm 9 (n=22)
Lupus nephritis	21	6386 \pm 2817 (n=21)	827 \pm 374 (n=21)	92 \pm 123 (n=21)	17 \pm 11 (n=21)	46 \pm 15 (n=21)	7 \pm 6 (n=21)
SLE alone	20	4715 \pm 2098 (n=20)	837 \pm 491 (n=20)	41 \pm 67 (n=20)	20 \pm 13 (n=20)	56 \pm 28 (n=20)	9 \pm 7 (n=20)

Data are reported as mean \pm SD. Numbers in parentheses indicate the number of patients with available data.

*Compared with diffuse NPSLE, p<0.05.

NPSLE, neuropsychiatric SLE; WBC, white blood cell.

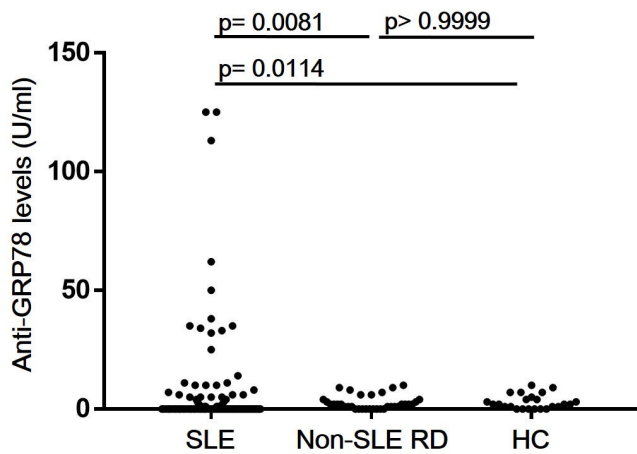


Figure 1 Serum anti-GRP78 levels in SLE, non-SLE rheumatic diseases (non-SLE RD) and healthy controls (HC). Statistical analysis was performed by Kruskal-Wallis test with Dunn's multiple comparison.

Serum anti-GRP78 levels were not correlated with white blood cell counts ($r=-0.02710$, $n=117$), lymphocyte counts ($r=-0.08538$, $n=107$), serum anti-DNA antibodies ($r=-0.00645$, $n=118$), serum anti-ribosomal P antibodies ($r=0.0122$, $n=129$), serum C3 ($r=-0.05077$, $n=113$), serum C4 ($r=-0.06244$, $n=113$) or CH50 ($r=-0.1112$, $n=114$) in patients with SLE. Moreover, serum anti-GRP78 levels were not correlated with serum IL-6 ($r=0.1141$, $n=88$) or CSF IL-6 ($r=0.1984$, $n=87$) in patients with NPSLE (diffuse and focal).

Relationship between BBB integrity and anti-GRP78 in patients with NPSLE patients

Next experiments explored the relationship of serum anti-GRP78 with BBB damages. As is consistent with

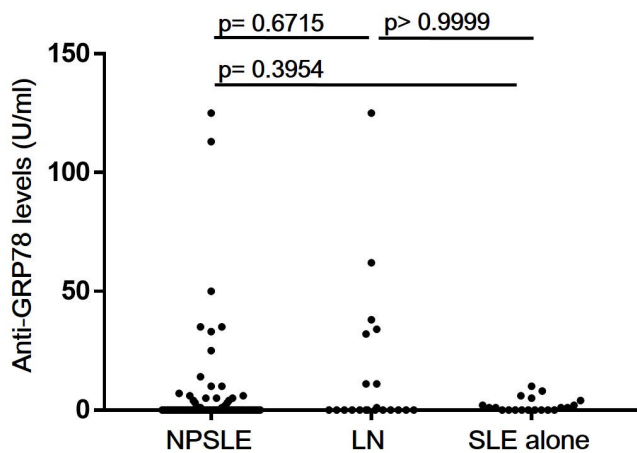


Figure 2 Serum anti-GRP78 levels in neuropsychiatric SLE (NPSLE), lupus nephritis (LN) and SLE without NPSLE or LN (SLE alone). Statistical analysis was performed by Kruskal-Wallis test with Dunn's multiple comparison.

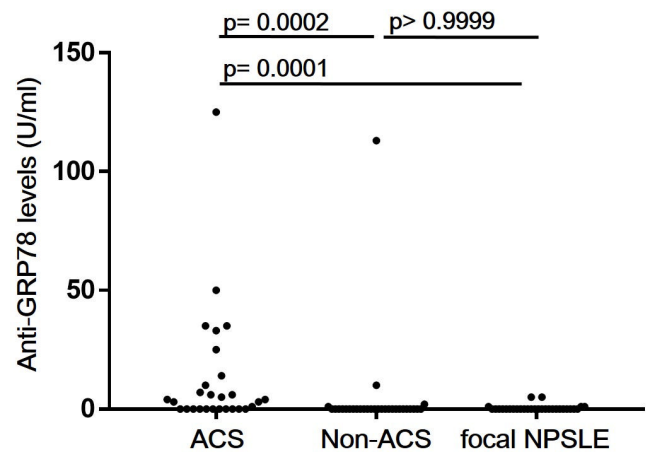


Figure 3 Serum anti-GRP78 levels in acute confusional state (ACS), non-ACS diffuse neuropsychiatric SLE (NPSLE) and focal NPSLE. Non-ACS diffuse NPSLE includes anxiety disorder, cognitive dysfunction, mood disorder and psychosis. Statistical analysis was performed by Kruskal-Wallis test with Dunn's multiple comparison.

previous studies,⁷ Q albumin values were significantly elevated in ACS (12.63 ± 12.07 (mean \pm SD)) compared with those in non-ACS diffuse NPSLE (4.75 ± 4.37) or in focal NPSLE (3.50 ± 2.93) (figure 4). Of note, anti-GRP78 levels were significantly correlated with Q albumin ($r=0.294$, $p=0.0054$) in 88 patients with NPSLE. These results confirm that the elevation of serum anti-GRP78 levels is associated with the damages of BBB in NPSLE.

Serum anti-N in patients with NPSLE and those with LN

It should be noted that there was no significant difference in serum anti-GRP78 levels between NPSLE and LN. When the mean+2SD value of serum anti-GRP78 (9.0 U/mL) in HC was set as the upper limit, serum anti-GRP78 levels exceeded this upper limit in 10 patients with diffuse NPSLE and in seven patients with LN. In order to examine why these seven patients with LN did not develop NPSLE, serum anti-N levels were compared between the 10 patients with diffuse NPSLE and the seven patients with LN. As shown in figure 5, serum anti-N levels were significantly higher in the 10 patients with diffuse NPSLE than those in the seven patients with LN. These results suggest that lower serum levels of anti-N might not be enough to result in the elevation of CSF levels of anti-N to cause neuronal damages despite the BBB breakdown due to anti-GRP78 in patients with LN. Thus, the elevation of neuron-reactive antibodies in sera in addition to the BBB damages mediated by anti-GRP78 is considered to be necessary for the development of diffuse NPSLE.

DISCUSSION

The importance of breakdown of BBB in the development in NPSLE, especially in ACS, has been well appreciated.^{7,19} However, the mechanism of BBB damages still

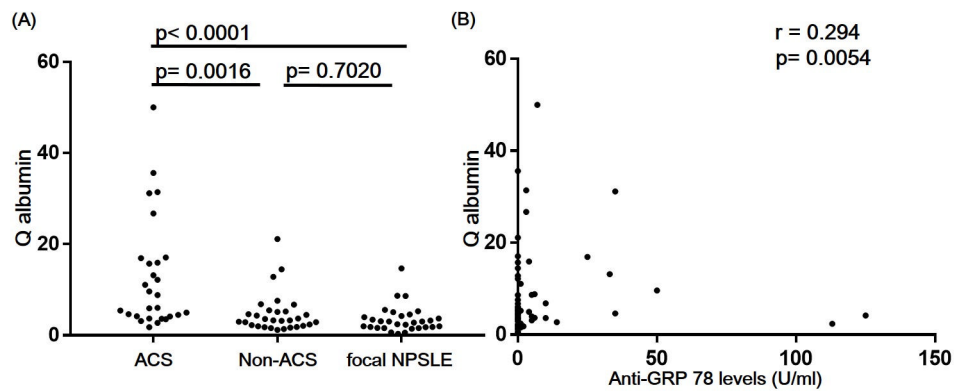


Figure 4 Blood–brain barrier integrity and its relationship with serum anti-GRP78 levels in patients with NPSLE. (A) Q albumin in acute confusional state (ACS), non-ACS diffuse neuropsychiatric SLE (NPSLE) and focal NPSLE. Statistical analysis was performed by Kruskal-Wallis test with Dunn's multiple comparison. (B) Correlation between Q albumin and serum anti-GRP78 levels in 88 patients with NPSLE (focal+diffuse). Statistical analysis was performed by Spearman's rank correlation test.

remains unclear. NMO or NMOSD is an inflammatory disease in which anti-AQP4 antibodies play a crucial role.⁹ Anti-AQP4 antibodies are produced mainly in the periphery, whereas they target astrocytes behind the BBB.⁹ Recent studies have discovered the antibodies which result in the decreased claudin5 protein expression of brain microvascular endothelial cells and the enhanced extravasation of albumin, IgG and fibrinogen.¹⁵ Analysis with unbiased membrane proteomics identified GRP78 as the target of these antibodies.¹⁵ More importantly, it was demonstrated that repeated administration of anti-GRP78 caused extravasation of IgG into the brain of mice.¹⁵ It is thus suggested that anti-GRP78 antibodies might promote transit of anti-AQP4 through BBB and result in NMOSD attacks.¹⁵

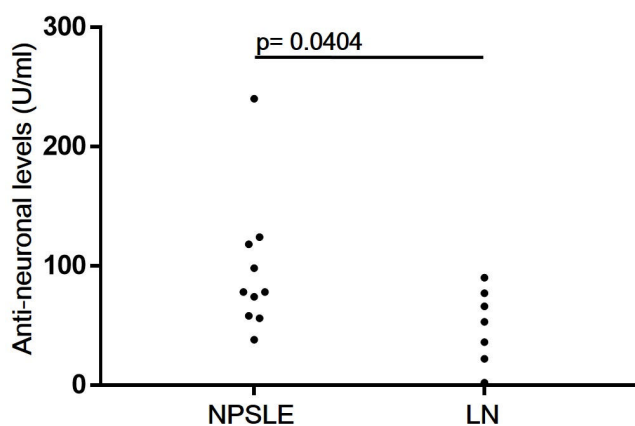


Figure 5 Serum anti-neuronal antibodies (anti-N) in patients with positive serum anti-GRP78. Patients with serum anti-GRP78 exceeding the mean+2 SD of healthy control group (9.0 U/mL) were defined as positive, including 10 patients with diffuse neuropsychiatric SLE (NPSLE) and in seven patients with lupus nephritis (LN). Serum anti-N levels were compared between these two groups. Statistical analysis was performed by Mann-Whitney U test.

SLE is a chronic inflammatory disease characterised by the expression of a variety of autoantibodies.¹⁶ In the present study, we have disclosed that serum anti-GRP78 levels in patients with SLE were elevated compared with those in non-SLE RD or in HC. More importantly, serum anti-GRP78 levels were significantly higher in ACS than those in non-ACS diffuse NPSLE or in focal NPSLE. Since the frequency of ACS in diffuse NPSLE is higher than that in previous reports,²⁰ it is possible that serum anti-GRP levels might be higher in our patients with diffuse NPSLE than expected. Notably, serum anti-GRP78 levels were significantly correlated with Q albumin in NPSLE. The results have therefore demonstrated that anti-GRP78 antibodies in the systemic circulation play a pivotal role in the damages of BBB in ACS. It should be noted, however, that serum anti-GRP78 levels exceeded the upper limit of normal in only 10 of 58 patients with diffuse NPSLE in the present study. It is therefore possible that there might be such autoantibodies other than anti-GRP78 that might cause damages to the BBB. In this regard, recent studies have disclosed that serum anti-Sm levels were significantly correlated with Q albumin in patients with NPSLE.²¹ It is thus suggested that anti-Sm might be also involved in BBB damages. Further studies would be required to determine whether there might be other autoantibodies that result in BBB damages as well as to explore how anti-Sm lead to BBB damages.

It has been demonstrated that cells in the CNS, including neurons, express GRP78.²² It is therefore possible that anti-GRP78 might affect the functions of neurons, leading to the development of neuropsychiatric manifestations. However, we could not detect anti-GRP78 antibodies in CSFs from 12 patients with ACS whose sera were positive for anti-GRP78 in the present study (data not shown). It is thus unlikely that anti-GRP78 might directly influence neurons in these patients with NPSLE.

A previous study demonstrated that anti-GRP78 antibodies were detected in the peripheral blood of normal healthy individuals.¹¹ Another study showed that

anti-GRP78 antibodies were present in 63% of sera from 400 patients with RA.²³ In the present study, we could not detect anti-GRP78 in sera either from most of 35 patients with non-SLE RD, including RA, or from 24 healthy individuals. This discrepancy might be due to the lack of manipulation to subtract non-specific binding of IgG to the plates.^{11 23} We have previously demonstrated that serum samples frequently express antibodies to carrier proteins or proteins used for overcoating the plates in ELISA.^{24 25} Therefore, subtracting the binding activity to these carrier proteins or overcoated proteins would be mandatory in order to exactly determine the specific activities of antibodies to the antigens of interest.^{24 25} Since no such manipulation of subtraction of non-specific bindings was carried out in the previous studies,^{11 23} it is doubtful that anti-GRP78 antibodies might present in sera from patients with RA or from healthy individuals.

It should be pointed out that serum anti-GRP78 levels were elevated in a fraction of patients with LN irrespective of LN histological typing (data not shown). It was thus possible that these seven patients with LN might have damages in BBB function, although they did not show NPSLE. Interestingly, the results in the present studies demonstrated that serum anti-N levels in the 10 patients with diffuse NPSLE with elevated serum anti-GRP78 were significantly higher than those in the seven anti-GRP78-positive patients with LN. It is therefore suggested that the seven patients with LN did not develop NPSLE due to the paucity of neuron-reactive autoantibodies in the systemic circulation. In this regard, previous studies demonstrated that the presence of anti-NMDA receptor antibodies in sera alone did not cause neuronal damages in mice until the breach of BBB was induced.⁵ Taken together, it is concluded that both the presence of neuron-reactive antibodies in sera and the breakdown of BBB are required for the development of diffuse NPSLE.

Of note, previous studies disclosed that abnormalities on MRI, serum IL-6 and serum anti-Sm significantly reduced the survival of patients with ACS.²⁶ In our study, 4 patients out of 8 patients with ACS with positive anti-GRP78 died, whereas 5 patients out of 20 patients with ACS with negative anti-GRP78 showed fatal outcome. Although the difference in mortality did not reach statistical significance in the present study, further studies with a larger number of patients would be required to identify such factors that would affect the outcome of patients with ACS, including serum anti-GRP78.

In summary, the present study has demonstrated that serum anti-GRP78 levels were significantly elevated in ACS diffuse NPSLE. Moreover, serum anti-GRP78 levels were significantly correlated with Q albumin in NPSLE, suggesting that anti-GRP78 antibodies might contribute to the development of ACS through the damages of BBB. Finally, serum anti-GRP78 levels were elevated in a fraction of patients with LN who did not show the elevation of serum anti-N. The results therefore are consistent with the previous observation that both the presence of

neuron-reactive antibodies in sera and the breakdown of BBB are required for the development of diffuse NPSLE.⁵

Contributors SH had full access to all of the data in the study and takes responsibility for the decision to submit for publication. SH designed the study and participated in experimental procedures, data collection, data analysis, data interpretation, and manuscript drafting and critical revising. YM contributed to data collection, data analysis, data interpretation, and manuscript drafting and critical revising. YA and TN contributed to data analysis and interpretation, and manuscript critical revising. SH and YM agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All the authors read and approved the final version of the manuscript to be published.

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Competing interests None declared.

Patient consent Obtained.

Ethics approval This study was approved by the institutional ethical committee of Teikyo University School of Medicine and that of Kitasato University School of Medicine (Ref. No. B09-55).

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