cerebrospinal fluid (CSF) has been reported to be associated with diffuse psychiatric/neuropsychological SLE, no significant correlation has been found between serum anti-NR2 antibody positivity and cognitive dysfunction [13-17]. In contrast, serum anti-NR2 antibody has been associated with depressive mood [15, 18]. Anti-NR2 antibody breaching the blood-brain barrier (BBB) can cause neuronal damage via an apoptotic pathway [12, 19]. Although the existence of anti-NR2 antibody in CSF is an important factor in neuronal damage in SLE, it remains unknown why the titre of serum anti-NR2A antibody is similar in SLE patients with and without neuropsychiatric symptoms. Additionally, the relationship between serum anti-NR2 antibody and non-nervous tissue damage such as serositis, nephritis and cytopenia has not been investigated in detail.

Each NR2 subunit consists of a large extracellular amino-terminal domain (ATD), a bilobed agonist-binding domain, a transmembrane domain and an intracellular C-terminal domain. The ATD is composed of approximately the first 350 amino acids of the protein [20]. Additionally, the ATD interacts with various extracellular allosteric modulators, such as zinc for NR2A, and plays an important role in fine-tuning the functional properties of NMDAR [20]. In ELISA, anti-NR2 antibody has been reported to react against the peptide DWEYSVWLSN [12, 17, 19]. DWEYS was reported as residues 283-287, which are included in the ATD [12, 20] of NR2A/2B. However, the actual sequence of residues 283-287 is DWDYS in NR2A and DEWDY in NR2B, according to the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). Asp 283 in NR2A and Glu 284 in NR2B are necessary for binding zinc [21]. Zinc binding to the NR2A/B ATD modulates intracellular Ca^{2+} signalling [21]. Therefore, we thought that the sequence near residue 283 must be an important region of each subunit. This would make DWDYS peptide more specific than DWEYS peptide in NR2A, even though most reports that have measured anti-NR2 antibody by ELISA in SLE patients have used DWEYS peptide.

In this report, we established a method to detect anti-NR2A antibody by ELISA using either DWEYS or DWDYS peptide as autoantigen. Additionally, we analysed the relationship between anti-NR2A antibody and various organ involvement in patients with SLE.

Patients and methods

Patients

This retrospective study included patients admitted to our hospital from January 2000 to December 2009. These patients were diagnosed with SLE based on the classification criteria of the ACR [22]. To evaluate cross-reactivity between dsDNA and NR2A, only patients with anti-dsDNA antibody were enrolled. All 107 enrolled patients suffered from symptoms associated with SLE on admission. Clinical data were obtained from medical records. Sera from all patients admitted to our institute were collected and stored. Sixty-eight patients were admitted for first treatment. The other 39 patients were recurrence and received intermediate/maintenance-dose steroid and/or immunosuppressive agents. The present study was approved by the ethical committee of the Institute of Rheumatology, Tokyo Women's Medical University in accordance with the Declaration of Helsinki.

Data collection

Disease activity was assessed using SLEDAI for each patient on admission [23]. Nephritis was defined as Class III, IV or V (proliferative, membranous or membranoproliferative) according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification [24, 25]. Renal biopsy was performed in 97 patients. The biopsy results prior to 2003 were reviewed again and classified by ISN/RPS 2003 classification. Neuropsychiatric SLE (NPSLE) was divided into the 19 neuropsychiatric syndromes classified by ACR [26]. Diffuse CNS form, focal CNS form and peripheral nervous system (PNS) form were defined as diffuse psychiatric/neuropsychological syndromes, neurological syndromes and PNS syndromes classified by ACR, respectively. The diagnosis of NPSLE was based on several investigations, such as neurological examination, brain/spinal cord MRI, electroencephalogram, CSF, nerve conduction examination, psychiatric interview and the short battery of neuropsychological tests that the ACR committee recommended [26, 27]. Cognitive function was evaluated in all patients except those with disturbance of consciousness or poor general condition. Other possible aetiologies of NPSLE, such as infection and drugs, were excluded. Laboratory tests included evaluation of complete blood count (leucocyte, haemoglobin and platelet) and immunological markers [CRP and complement components (C3 and C4)]. Anti-dsDNA antibody was measured by RIA (normal value is <6 IU/ml). Antibodies to U1-snRNP, Sm and SSA were measured by double ID.

Measurement of anti-NR2A antibody

We used the two peptides, DWEYSVWLSN (DWEYS peptide) and ISVSYDDWDYSLE (DWDYS peptide), to measure anti-NR2A antibody by ELISA. DWEYSVWLSN is the peptide most frequently used in previous reports [12, 17, 19]. On the other hand, ISVSYDDWDYSLE is the sequence of NR2A residues 277-289, according to the NCBI. Each peptide was a highly purified synthetic peptide conjugated to BSA as antigen (Serologicals, Norcross, GA, USA). Ninety-six-well microtitre plates (IWAKI, Tokyo, Japan) were coated with 0.5 μg of synthetic peptide BSA or only BSA in 100 μl of PBS (pH 7.4) per well overnight at 4°C. The wells were blocked with Block Ace (Dainippon, Osaka, Japan) in PBS for 2 h at 37°C. Samples (100 μl) diluted 1 : 1000 in 10% Block Ace were added to each well and incubated for 1 h at 37°C. The plates were then washed with PBS-Tween and further incubated with goat anti-human IgG conjugated with peroxidase (MBL, Aichi, Japan) for 1 h at 37°C. The plates were washed again with PBS-Tween. Tetramethyl benzidine (Sigma-Aldrich, St Louis, MO, USA; 100 μl) was

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added to each well and incubated at 37°C. After 15 min, the reaction was stopped by the addition of 2 N H_2SO_4. Optical density (OD) at 450 nm was measured using a Titertek Multiscan Plus ELISA reader (Bio-Rad, Philadelphia, PA, USA). The OD value of anti-NR2A antibody was estimated by subtracting from the OD value of BSA-conjugated peptide the OD value of BSA alone. The intra- and inter-assay variances (coefficients of variation) for anti-NR2A were 3.5 and 15.5%, respectively.

Statistical analysis

Statistical analyses were performed by using the chi-square test to compare frequencies, the *t*-test to compare mean values and the Mann-Whitney U-test to compare median values. Correlation coefficients were calculated as Pearson's correlation coefficient or Spearman's rank correlation coefficient. Multiple linear regression analysis was performed to evaluate the association between anti-NR2A antibody and the various organ involvements. To analyse multiple regression appropriately, we used a forward stepwise model, which involved starting with no independent variables and trying out the variables one by one. First, the most statistically significant independent variable was included in the model. Next, among the other independent variables, the most statistically significant variable was added in the model. This process was repeated and the variables were added one by one in the stepwise model until the *P*-values of the variables were >0.1. The data were analysed using JMP software (SAS Institute, Cary, NC, USA). $P < 0.05$ indicated statistical significance. Bonferoni correction was used for multiple comparison.

Results

Comparison of the measurement of serum anti-NR2A antibody with each peptide

Figure 1A shows the relationship between the OD value and dilution of sera using either DWEYS or DWDYS peptide and the high-titre positive sample. The OD value was higher with DWDYS peptide. Figure 1B shows the OD value of serum anti-NR2A antibody with either DWEYS or DWDYS peptide. Samples were collected from 20 SLE patients. The median OD (range) value using DWDYS peptide, 0.407 (0.293–0.616), was significantly higher than with DWEYS peptide, 0.126 (0.079–0.247; $P < 0.0001$). Additionally, as shown in Fig. 1C, the OD values using the two peptides correlated significantly with each other ($r = 0.94$, $P < 0.0001$). These results demonstrate that the use of DWDYS peptide was more sensitive than DWEYS peptide in detecting anti-NR2A antibody in ELISA.

Fig. 1 Comparison of the measurement of serum anti-NR2A antibody using each peptide. (**A**) Relationship between the OD value and dilution of sera using either DWEYS or DWDYS peptide. (**B**) Serum anti-NR2A antibody was measured by ELISA using DWEYS and DWDYS peptides. Samples were collected from 20 SLE patients. (**C**) Correlation between the OD value using DWDYS peptide and the OD value using DWEYS peptide.

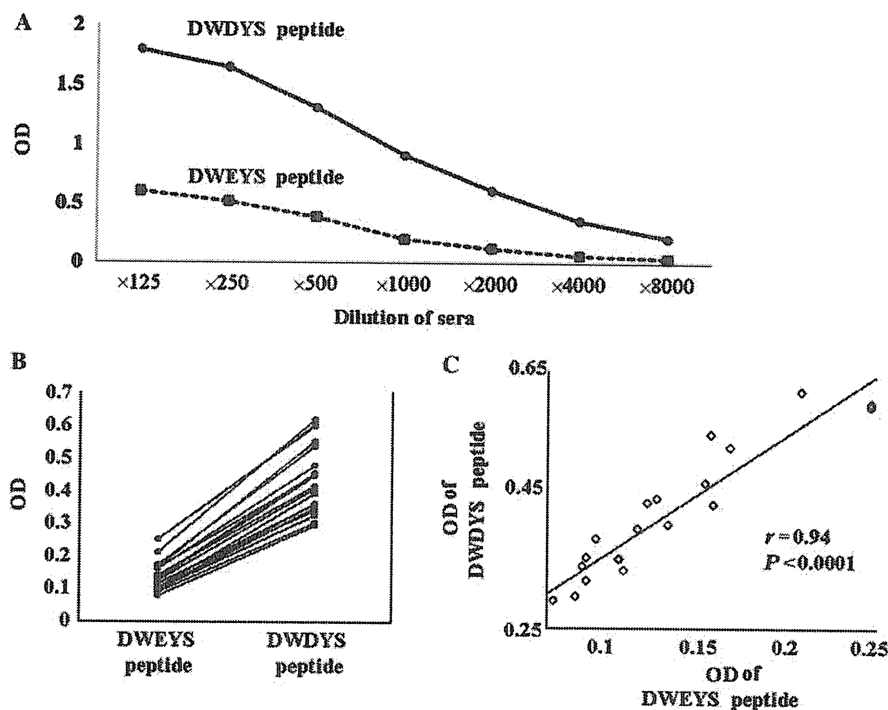

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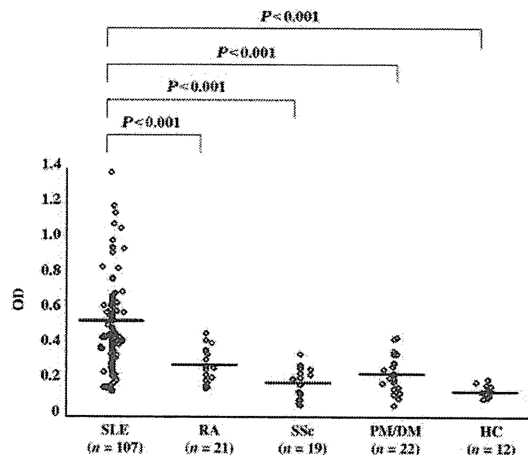
FIG. 2 Measurement of serum anti-NR2A antibody in SLE and non-SLE. Bar indicates the median OD value in each subset. HC: healthy controls.



Measurement of serum anti-NR2A antibody in SLE and non-SLE patients

Figure 2 shows the OD value of serum anti-NR2A antibody in both SLE and non-SLE patients. One hundred and seven patients were included in the SLE subset. The non-SLE subset included 21 patients with RA, 19 patients with SSc, 22 patients with PM/DM and 12 healthy controls (HC). The diagnosis was based on the classification criteria of the ACR in RA and SSc, and the criteria of Bohan and Peter in PM/DM, respectively [28-30]. The mean age and the frequency of females were matched between SLE subset and HC. The median OD (interquartile range) values were 0.449 (0.327-0.622), 0.275 (0.19-0.358), 0.141 (0.096-0.24), 0.225 (0.151-0.312) and 0.146 (0.122-0.177) in SLE, RA, SSc, PM/DM and HC, respectively. There was a strikingly significant difference between SLE and each non-SLE subset ($P < 0.0001$).

Demographic and clinical characteristics of patients with SLE

All patients with SLE were Japanese, except two patients who were Korean. Table 1 shows the demographic and clinical characteristics from the first examinations on admission. The patients were 37 (14) years old [mean (S.D.)]. The frequency of females was 97%. The median score (interquartile range) of the SLEDAI was 12 (8-19). The frequency of each clinical feature was as follows: malar erythema 22%, arthritis 38%, serositis 14%, nephritis 43% (proliferative 54%, membranoproliferative 22% and membranous 24%) and NPSLE 22% (cerebrovascular disease 33%, cognitive dysfunction 21%, psychosis 17%, seizure and polyneuropathy 13% and others 4%). Combined NP forms, such as the focal and diffuse CNS forms, were revealed in six patients with SLE. The positive frequency of each antibody was as follows: anti-dsDNA 100%, anti-U1 snRNP 36%, anti-Sm 21% and anti-SSA 54%.

TABLE 1 Demographic and clinical characteristics of 107 patients with SLE

Age, mean (S.D.), years	37 (14)
Gender: female, *n* (%)	104 (97)
SLEDAI, median (interquartile range)	12 (8-19)
Malar erythema, *n* (%)	24 (22)
Arthritis, *n* (%)	41 (38)
Serositis, *n* (%)	15 (14)
Nephritis, *n* (%)	46 (43)
NPSLE, *n* (%)	24 (22)
Antibody positive, *n* (%)	
Anti-dsDNA	107 (100)
Anti-U1 snRNP	39 (36)
Anti-Sm	22 (21)
Anti-SSA	58 (54)

Comparison of clinical characteristics between anti-NR2A antibody-positive and -negative SLE patients

Table 2 shows the comparison of clinical characteristics between anti-NR2A antibody-positive (*P* group) and antibody-negative SLE patients (*N* group). Anti-NR2A antibody was measured by ELISA using DWDYS peptide. Anti-NR2A antibody positivity was defined as an OD > 0.62, which was the mean (4 S.D.) in the non-SLE subset. There were 27 (25%) and 80 (75%) patients in the *P* and *N* groups, respectively. Twenty-one items were compared in clinical characteristics between the *P* and *N* groups. The significant *P*-value was 0.0024, adjusted by Bonferoni correction. The mean age and the frequency of females showed no significant differences. The SLEDAI score and the frequencies of serositis and nephritis were higher in the *P* group, although there was no statistical significance. The frequencies of NPSLE were significantly higher ($P = 0.0002$, and odds ratio 5.8 in the *P* group). Additionally, the frequencies of diffuse CNS form and focal CNS form were higher ($P = 0.036$ and 0.01, and odds ratio 3.5 and 5.3, respectively). Leucocyte count and haemoglobin were lower ($P = 0.021$ and 0.0008, respectively) in the *P* group. The values of CRP, complement and anti-dsDNA antibody showed no significant differences between the two groups. Positivity for anti-U1 snRNP, anti-Sm and anti-SSA antibodies showed no significant difference between the two groups.

Correlation coefficients between the anti-NR2A and anti-dsDNA antibody and clinical parameters

As shown in Table 3, the correlation coefficients between parameters were established in SLE patients. The parameters included the OD values for anti-NR2A antibody and anti-dsDNA antibody, SLEDAI and laboratory markers (C3, leucocyte, haemoglobin and platelet). No correlations were found between anti-NR2A antibody and anti-dsDNA antibody or C3, although significant correlations were

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TABLE 2 Comparison of clinical characteristics between anti-NR2A antibody-positive and -negative SLE patients

Variables	Anti-NR2A positive ($n=27$)	Anti-NR2A negative ($n=80$)	P-value	Odds ratio (95% CI)
Age, mean (S.D.), years	34 (16)	34 (14)	0.96	
Gender: female, n (%)	27 (100)	77 (96)	0.55	
SLEDAI, median (interquartile range)	16 (9–30)	11 (8–15)	0.023	
Malar erythema, n (%)	8 (30)	16 (20)	0.3	
Arthritis, n (%)	13 (48)	28 (35)	0.22	
Serositis, n (%)	7 (26)	8 (10)	0.039	3.2 (1.0, 9.7)
Nephritis, n (%)	17 (63)	29 (36)	0.015	3.0 (1.2, 7.4)
NPSLE, n (%)	13 (48)	11 (14)	0.0002	5.8 (2.2, 15.6)
Diffuse CNS form	6 (22)	6 (8)	0.036	3.5 (1.0, 12.1)
Focal CNS form	7 (26)	5 (6)	0.01	5.3 (1.5, 18.3)
PNS form	4 (15)	3 (4)	0.066	4.5 (0.9, 21.4)
Leucocytes/μl, median (interquartile range)	3800 (2600–5100)	4650 (3400–6475)	0.021	
Haemoglobin, median (interquartile range), g/dl	10.1 (8.3–11.3)	11.5 (9.4–12.7)	0.0008	
Platelets $\times 10^4$/μl, median (interquartile range)	22.5 (14.0–31.1)	20.2 (14.7–26.8)	0.34	
CRP, median (interquartile range), mg/dl	0.3 (0.1–1.0)	0.1 (0–0.8)	0.18	
C3, median (interquartile range), mg/dl	53 (39–72)	57 (38–77)	0.73	
C4, median (interquartile range), mg/dl	5 (3–14)	6 (3–13)	0.98	
Anti-dsDNA Ab, median (interquartile range), IU/ml	58 (19–310)	44 (19–124)	0.32	
Antibody positivity, n (%)				
Anti-U1 snRNP	8 (30)	31 (39)	0.87	
Anti-Sm	4 (15)	18 (23)	0.58	
Anti-SSA	18 (67)	40 (50)	0.13	

TABLE 3 Correlation coefficients between the OD values of anti-NR2A and anti-dsDNA antibodies and clinical parameters

Variables	Anti-NR2A antibody		Anti-dsDNA antibody	
	r_s	P-value	r_s	P-value
vs SLEDAI	0.19	0.049	0.3	0.0019
vs anti-dsDNA antibody titre	0.16	0.095	–	–
vs C3 value	−0.11	0.24	−0.38	<0.0001
vs leucocyte count	−0.31	0.001	−0.24	0.014
vs haemoglobin count	−0.42	<0.0001	−0.15	0.12
vs platelet count	0.047	0.63	−0.21	0.027

r_s: correlation coefficient estimated by Spearman's rank correlation coefficient.

found between anti-NR2A antibody and leucocyte count ($r_s = -0.31$, $P = 0.001$) and haemoglobin ($r_s = -0.42$, $P < 0.0001$).

On the other hand, significant correlations were found between anti-dsDNA antibody and SLEDAI ($r_s = -0.3$, $P = 0.019$) and between anti-dsDNA antibody and C3 ($r_s = -0.38$, $P < 0.0001$). Additionally, mild but significant correlations were found between anti-dsDNA antibody and leucocyte ($r_s = -0.24$, $P = 0.014$) and platelet counts ($r_s = -0.21$, $P = 0.027$).

Association between anti-NR2A antibody and various organ involvements and clinical parameters

Multiple linear regression analysis was performed with a forward stepwise adjustment to evaluate the association between anti-NR2A antibody, various organ involvements and clinical parameters. Anti-NR2A antibody positivity was the dependent variable. Independent variables included the following: serositis, nephritis, NPSLE, leucocyte, haemoglobin, C3 and anti-dsDNA antibody. Stepwise adjustment revealed that the variables of anti-dsDNA antibody and serositis were excluded in the stepwise model because the P-value was 0.6381 and 0.5884, respectively. Actually, nephritis, NPSLE, leucocyte, haemoglobin and C3 were used as independent variables in the multivariate analysis. As shown in Table 4, NPSLE was the most significant independent variable ($P = 0.0008$). Blood counts such as leucocyte and haemoglobin were

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TABLE 4 Association between anti-NR2A antibody, various organ involvements and clinical parameters

Variables	Odds ratio (95% CI)	*P*-value
Nephritis	2.6 (0.9, 8.1)	0.078
NPSLE	7.8 (2.4, 27.8)	0.0008
Leucocyte, per unit	0.9996 (0.9993, 0.9999)	0.0095
Haemoglobin, per unit	0.7 (0.52, 0.93)	0.013
C3, per unit	1.03 (1.002, 1.05)	0.04

Multiple linear regression analysis was performed with stepwise adjustment. Dependent variable was anti-NR2A antibody positivity. Independent variables included nephritis, NPSLE, leucocyte, haemoglobin and C3.

also significant variables ($P = 0.0095$ and 0.013, respectively).

Discussion

We have demonstrated clinical manifestations in serum anti-NR2A antibody-positive patients with SLE. For the first time, we have analysed the association between anti-NR2A antibody and various organ involvements in detail. Additionally, we measured anti-NR2A antibody using a different peptide antigen from that used in all previous reports [12, 14–18] except one [13]. We considered that the sequence near Asp 283 must be important because Zn binding to Asp 283 modulates intercellular Ca^{2+} signalling in cells expressing NR2A [21]. The actual sequence of residues 277–291 is ISVSYDDWDYSLEAR, according to the NCBI (http://www.ncbi.nlm.nih.gov/), not DWEYSVWLSN, as previously reported. The median anti-NR2A antibody OD value was significantly higher ($P < 0.0001$) and the range more broad in the ELISA system using the peptide ISVSYDDWDYSLE compared with DWEYSVWLSN, although the OD values from the two peptides correlated significantly with each other ($r = 0.94$, $P < 0.0001$). On the other hand, no correlation has been previously found between anti-NR2A antibody positivity and NPSLE, such as with the focal CNS form, although serum anti-NR2A antibody has been associated with depressive mood [15, 18]. However, our results show that the frequency of NPSLE, including both diffuse and focal CNS forms, was significantly higher ($P = 0.0002$) in patients with serum anti-NR2A antibody. Additionally, NPSLE was the most significantly associated clinical parameter ($P = 0.0008$) with anti-NR2A antibody, as calculated by multiple linear regression analysis. For these reasons, we considered the ISVSYDDWDYSLE peptide more accurate and specific than DWEYSVWLSN in measurement of anti-NR2A antibody using ELISA. The differences in the peptides used in ELISAs may have contributed to the differences between the results of the present study and previous reports.

Husebye *et al.* [13] used SVSYDDWDYSLEARV (residues 278–292 of NR2A) in ELISA. This peptide is almost identical to the peptide we used. They reported that sera of 34 (31%) out of 109 SLE patients reacted specifically with the NR2A peptide, and no correlation was found between the presence of anti-NR2A and anti-dsDNA antibodies. These findings are consistent with our observations. On the other hand, they found no significant correlation between anti-NR2A antibody and NPSLE because the number of patients with NPSLE was small, 6 (5.5%). In contrast, the number of patients with NPSLE was 24 (22%) in our study. Thus, the differences in the study populations could explain the differences in the results regarding correlations between anti-NR2A antibody and clinical manifestations. In the present study, the SLEDAI score was higher in patients with anti-NR2A antibody than those without anti-NR2A antibody, although the values of anti-dsDNA antibody and complement showed no significant differences between patients with and without anti-NR2A antibody. NPSLE was the clinical parameter most significantly associated with anti-NR2A antibody. The SLEDAI result could be related to the frequency of NPSLE in patients with anti-NR2A antibody. The present study demonstrates that the anti-NR2A antibody could be associated with the complications of NPSLE.

No significant correlation was found between anti-NR2A antibody and previously used markers of SLE, such as anti-dsDNA antibody and complement, although the SLEDAI score was significantly higher ($P = 0.023$) in patients with anti-NR2A antibody. On the other hand, anti-dsDNA antibody was significantly correlated with the value of C3 in the present study. Immune aggregates are present at sites of injury in glomeruli, as are complement components in LN. LN patients have the high-avidity anti-dsDNA antibodies that activate complement strongly, leading to complement-dependent cytotoxicity (CDC). Higher-avidity anti-dsDNA antibodies also occur in proliferative nephritis, and cationic antibodies appear to be more pathogenic [31]. On the other hand, Degiorgio *et al.* [12] demonstrated that anti-dsDNA antibody interacts with NMDAR and can signal neuronal death through an excitotoxic mechanism without inflammation or vasculopathy. However, not all anti-dsDNA antibodies are not able to cross-react with NR2. The present study found that the frequency of anti-NR2A antibody positivity was 25% in SLE patients. Taken together, the current and previous data suggest that anti-DNA antibodies that are able to cross-react with NR2A may damage cells not only via CDC but also via antibody-dependent cell-mediated cytotoxicity or an apoptotic pathway.

Only 48% of SLE patients with anti-NR2A antibody exhibited the NP manifestation (Table 2). Anti-NR2A antibody that reaches the CNS through the BBB is thought to potently damage neuronal cells [19]. Abbott *et al.* [32] described the two main mechanisms for BBB damage in SLE: microthrombi in cerebral vessels and immune-mediated attack of the endothelium. Anti-NMDAR antibody has been detected in paraneoplastic encephalitis associated with ovarian teratoma and is believed to be produced to cross-react with teratoma as an antigen [6]. Both the induction of anti-NR2A antibody in peripheral organs and the impairment of the BBB or

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blood–nerve barrier could be attributed to the establishment of NP syndrome in SLE patients. Since NMDAR is located in the CNS more than any other organ, the manifestations of CNS may be more apparent than those of PNS or a non-nervous organ in SLE patients with anti-NR2A antibody. On the other hand, the relationship between anti-NR2 antibody and non-nervous tissue involvement has not been analysed. NMDAR is also located on the surface of cells in non-neuronal tissues [9]. The present study found higher frequencies of nephritis and diminished counts of leucocytes and red blood cells in anti-NR2A antibody-positive SLE patients. Significant correlations were found between the OD values of anti-NR2A antibody and blood cell counts. Those observations indicate that anti-NR2A antibody may involve non-neuronal tissues.

In conclusion, serum anti-NR2A antibody could be associated with the complication of NP in patients with SLE. Anti-NR2A antibody may also be associated with the involvement of non-nervous tissues, such as blood cells and kidney. The use of peptides including DWDYS is preferable to DWEYS in ELISAs to detect anti-NR2A antibody.

Rheumatology key messages

- Serum anti-NR2A antibody could be associated with the complication of NPSLE.
- Anti-NR2A antibody may also be involved in damaging both nervous and non-nervous tissues.

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NR2-Reactive Antibody Decreases Cell Viability Through Augmentation of Ca^{2+} Influx in Systemic Lupus Erythematosus

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Objective. **Anti–*N*-methyl-D-aspartate (anti-NMDA) receptor subunit NR2–reactive antibody may play a crucial role in neuronal manifestations of systemic lupus erythematosus (SLE). However, how NR2-reactive antibody acts as a critical modulator of the NMDA receptor is unknown. This study was undertaken to investigate the biologic function of NR2-reactive antibody in patients with SLE.**

Methods. **The study included 14 patients with SLE, 9 of whom had NR2-reactive antibody. We analyzed the effects of NR2-reactive antibody on cell viability and intracellular Ca^{2+} level. We also investigated the efficacy of zinc as a modulator of the intracellular Ca^{2+} level in the presence of NR2-reactive antibody.**

Results. **There was a significant inverse correlation between the NR2-reactive antibody titer and cell viability ($R^2 = 0.67$, $P < 0.0001$; n = 23), and there was a significant association between the NR2-reactive antibody titer and the intracellular Ca^{2+} level in NR1/NR2a-transfected HEK 293 cells ($R^2 = 0.69$, $P < 0.0001$). Intracellular Ca^{2+} levels were significantly higher in cells incubated with IgG derived from NR2-reactive antibody–positive SLE patients than in those incubated with IgG derived from NR2-reactive antibody–negative SLE patients ($P = 0.0002$). The addition of zinc decreased the intracellular Ca^{2+} level in a dose-dependent manner. NR2-reactive antibody–positive SLE IgG weakened the efficacy of zinc as a negative modulator of the intracellular Ca^{2+} level.**

Conclusion. **Our findings indicate that NR2-reactive antibody decreases cell viability by Ca^{2+} influx in SLE through inhibition of the binding capacity of zinc.**

N-methyl-D-aspartate (NMDA) receptors are ligand-gated ion channels that play crucial roles in synaptic transmission and central nervous system (CNS) plasticity. The receptors are heterodimers of NMDA receptor subunits NR1, which bind glycine, and NR2 (NR2a, NR2b, NR2c, or NR2d), which bind glutamate (1). NMDA receptor dysfunction is implicated in multiple brain disorders, including stroke, chronic neurodegeneration, epilepsy, and schizophrenia (2–5). In contrast, NMDA receptor–reactive antibody is observed in various autoimmune disorders, and anti-NR1/NR2 antibody–associated encephalitis has recently been described by several researchers (6–8).

Systemic lupus erythematosus (SLE) is a multisystem inflammatory disorder characterized by the presence of autoantibodies directed against double-stranded DNA (dsDNA). Some anti-dsDNA antibodies cross-react with NR2 and damage neuronal cells via an apoptotic pathway (9). Not all anti-dsDNA antibodies cross-react with NR2 to the same degree. We previously found no association between the anti-dsDNA antibody titer and the NR2-reactive antibody titer in 107 patients with SLE (10). The frequency of serum NR2-reactive antibody positivity is ~30–40% in patients with SLE (10–14).

Associations between serum NR2-reactive antibody positivity and neuropsychiatric SLE (NPSLE) have been demonstrated in some previous studies (10,12,14). In contrast, other studies showed no significant association between serum NR2-reactive antibody positivity and cognitive dysfunction (11,13,15,16). DeGiorgio and

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colleagues have shown that NR2-reactive antibody breaching the blood–brain barrier can cause neuronal damage via an apoptotic pathway (9,17). The existence of NR2-reactive antibody in cerebrospinal fluid is an important factor in neuronal damage in SLE. However, how NR2-reactive antibody breaches the CNS through the blood–brain barrier and causes neuronal damage via an apoptotic pathway is not known. Recently, Faust and colleagues demonstrated that NR2-reactive antibody acts as a positive modulator of NMDA receptor–mediated synaptic responses and toxicity and preferentially binds to the open NMDA receptor pore (18). These results suggest that NR2-reactive antibody binding to NMDA receptor prolongs the open state, which would increase Ca^{2+} influx into cells.

Each NR2 subunit consists of a large extracellular amino-terminal domain, a bilobed agonist-binding domain, a transmembrane domain, and an intracellular C-terminal domain. The amino-terminal domain is composed of the first ~350 amino acids of the protein (19). Additionally, the amino-terminal domain interacts with various extracellular allosteric modulators, such as zinc in the case of NR2a/NR2b, and plays an important role in fine-tuning the functional properties of the NMDA receptor (19). In enzyme-linked immunosorbent assays (ELISAs), NR2-reactive antibody in patients with SLE can react against the peptide DWEYSVWLSN (9,16,17). DWEYS has been reported as the common sequence of residues 283–287 of NR2a/NR2b, which are included in the amino-terminal domain (9,19). However, the actual sequence of residues 283–287 is DWDYS in NR2a and DEWDY in NR2b, according to the NCBI (http://www.ncbi.nlm.nih.gov/). Asp^{283} in NR2a and Glu^{284} in NR2b are considered zinc-binding sites. Zinc binding to the NR2a/NR2b amino-terminal domain modulates NMDA receptor–mediated synaptic responses (20). Therefore, the sequence near residue 283 must be an important region of NR2. We speculated that NR2-reactive antibody could react with the zinc-binding site of NR2 to promote intracellular Ca^{2+} signaling and damage neuronal cells.

To determine the precise functions of NR2-reactive antibody in patients with SLE, we analyzed the effects of NR2-reactive antibody on cell viability and Ca^{2+} influx. We also investigated the efficacy of zinc as a modulator of intracellular Ca^{2+} levels in the presence or absence of NR2-reactive antibody.

MATERIALS AND METHODS

Materials. Patients were diagnosed as having SLE based on the American College of Rheumatology classification criteria (21). Sera were obtained from 9 NR2-reactive antibody–positive patients, 5 NR2-reactive antibody–negative patients, and 9 healthy controls. IgG was extracted and purified from sera by standard column-based methods. Each IgG sample obtained was used in every experiment. HEK 293 cells were purchased from RIKEN Cell Bank. The plasmid constructs pcDNA3.1-NR1, pcDNA3.1-NR2a, and pcDNA3.1 were generous gifts from Dr. Jon W. Johnson (University of Pittsburgh, Pittsburgh, PA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco BRL. Fulo-3 acetoxymethyl ester was obtained from Molecular Probes. NMDA was purchased from Sigma. Other chemicals were of the highest purity commercially available.

Measurement of NR2-reactive antibody. We used the peptide ISVSYDDWDYSLE to measure NR2-reactive antibody by ELISA. This peptide is the sequence of NR2a residues 277–289. Serum samples (100 μl) diluted 1:1,000 in 10% Block-Ace (Dainippon) were added to each well, as previously described (10).

Expression of NMDA receptors in HEK cells. HEK 293 cells were plated at a density of 2.5×10^4 cells in a 4-well dish. HEK 293 cells were grown in DMEM supplemented with 5% fetal bovine serum for 24 hours before transfection (22). We used rat NMDA receptor subunits cloned into expression vectors as previously described (23). Cells were transfected at a 1:3 ratio with NR1 and NR2a subunit expression vector by the calcium phosphate coprecipitation method, followed by further culture for an additional 24 hours (24).

Determination of cell viability. Cell viability was measured by MTT reduction colorimetric assays with minor modifications (23,25). As described above, HEK 293 cells were incubated for 24 hours after transfection. Cells were then cultured for another 24 hours in DMEM containing zinc at 1 μM, 10 μM, or 100 μM and purified IgG obtained from sera at 0.1 mg/ml. NR1/NR2a-transfected cells were then washed once with phosphate buffered saline (PBS) and incubated with 0.5 mg/ml MTT in PBS for 2 hours. NR1/NR2a-transfected cells were then solubilized by the addition of a lysis solution containing 99.5% isopropanol and 0.04M HCl. The amount of MTT formazan product was determined by measuring the absorbance at 550 nm on a microplate reader. Relative values were calculated as percentages above the value obtained in cells with empty vector (control group). Additionally, the background value obtained under cell-free conditions was subtracted from the total value.

Measurement of intracellular Ca^{2+} level. Intracellular Ca^{2+} was measured as previously described (24,26). After transfection, incubated cells were washed twice with recording medium containing 129 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 4.2 mM glucose, and 10 mM HEPES (pH 7.4), followed by incubation at 37°C for 1 hour in recording medium, which contained 60 nM Pluronic F-127, and 3 μM Fluo-3. Cells were washed with recording medium once, followed by settling for 1 hour in recording medium containing 0.1 mg/ml purified IgG obtained from sera. Confocal laser scanning microscopy was used to observe the intracellular free Ca^{2+} level. The medium was changed once more, followed by exposure to NMDA at 0.1 μM, 1.0 μM, 10 μM, 100 μM, or 1,000 μM. NMDA was prepared in recording medium immediately before each use. The calcium ionophore A23187 was then added at 10 μM to obtain the maximum fluorescence for

quantitative normalization. Fluorescence was recorded using an excitation wavelength of 488 nm. The data obtained were normalized to the basal level of fluorescence intensity in cells exposed to 10 μM A23187.

Evaluation of the effect of zinc on intracellular free Ca^{2+} levels. As described above, after transfection, HEK 293 cells were washed with recording medium, followed by incubation at 37°C for 1 hour in recording medium containing 60 nM Pluronic F-127 and 3 μM Fluo-3. Incubated cells were washed with recording medium once, followed by settling for 1 hour in recording medium containing 0.1 mg/ml purified IgG obtained from sera. The medium was changed once more. NMDA (10 μM) was added, followed by exposure to zinc at 0.01 μM, 0.1 μM, 1.0 μM, 10 μM, or 100 μM. Intracellular free Ca^{2+} was measured as described above.

Statistical analysis. The *t*-test was used to compare mean values, and the Mann-Whitney U test was used to compare median values. Regression analysis was performed when appropriate. Correlations were measured with Pearson's correlation coefficient. The data were analyzed with JMP software (SAS Institute). *P* values less than 0.05 were considered significant.

RESULTS

Association between anti-dsDNA antibody and NR2-reactive antibody in SLE patients. Serum NR2-reactive antibody was measured by ELISA, using the DWDYS peptide, which comprises residues 283–287 of NR2a (10). NR2-reactive antibody positivity was defined as an optical density (OD) of >0.62. This cutoff value was based on a mean ± SD OD of 0.62 ± 4 determined in 74 non-SLE serum samples, including samples from 21 patients with rheumatoid arthritis, 19 patients with systemic sclerosis, 22 patients with polymyositis/dermatomyositis, and 12 healthy controls. Anti-dsDNA antibody was measured by radioimmunoassay. (The normal value is <6 IU/ml.) Neuropsychiatric symptoms were observed in 5 (56%) of 9 patients with NR2-reactive antibody–positive SLE.

Five NR2-reactive antibody–negative patients were also enrolled in the study in order to evaluate the association between anti-dsDNA antibody and NR2-reactive antibody. There was no significant difference in anti-dsDNA antibody titer between the 9 patients with NR2-reactive antibody and the 5 patients without NR2-reactive antibody ($P = 0.20$). There was no statistically significant correlation between NR2-reactive antibody titer and anti-dsDNA antibody titer ($R^2 = 0.24$, $P = 0.09$; n = 14).

Association between NR2-reactive antibody titer and NMDA receptor–induced cell viability. To estimate the effect of NR2-reactive antibody in patients with SLE on cell viability, NR1/NR2a-transfected HEK 293 cells were incubated with healthy control IgG (0.1 mg/ml; n = 9), NR2-reactive antibody–negative SLE IgG (0.1 mg/ml; n = 5), or NR2-reactive antibody–positive SLE IgG (0.1 mg/ml; n = 9). Cell viability was calculated as the percentage above the value obtained in control cells that were transfected with empty vector alone. The concentration of NR2-reactive antibody is expressed as the OD value. Regression analysis was performed with NR2-reactive antibody as the independent variable and cell viability as the dependent variable. There was a statistically significant association between NR2-reactive antibody titer and cell viability ($R^2 = 0.67$, $P < 0.0001$; n = 23) (Figure 1A). There was no significant association

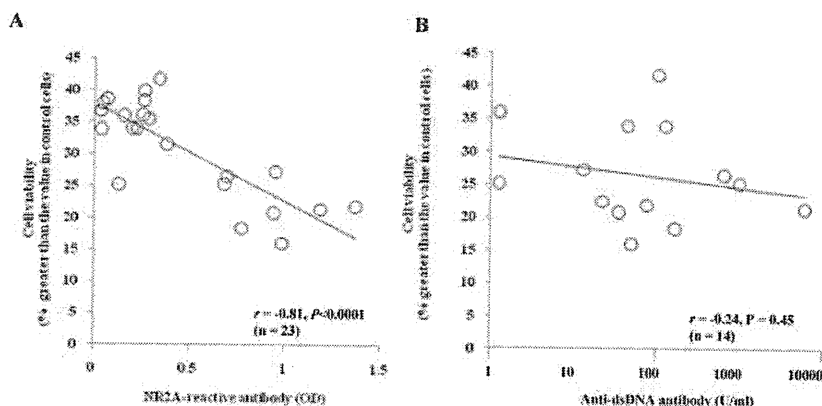

Figure 1. A, Significant association between *N*-methyl-D-aspartate receptor subunit NR2–reactive antibody titers and cell viability. **B,** Lack of a significant association between anti–double-stranded DNA (anti-dsDNA) antibody titers and cell viability. Values on the x-axis in **B** are the base 10 logarithm. Circles represent individual samples. OD = optical density.

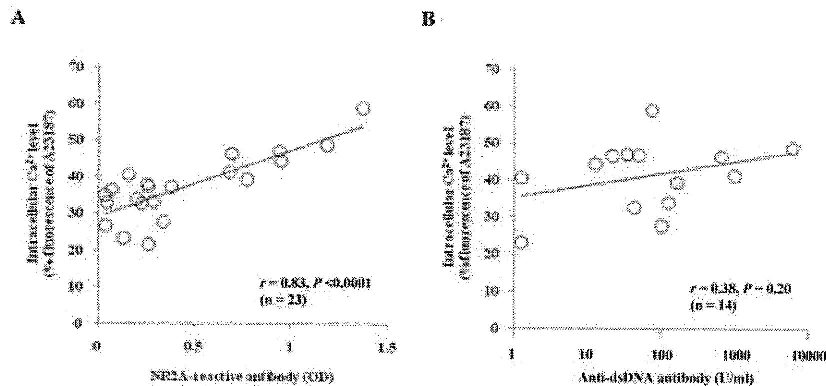

Figure 2. **A,** Significant association between NR2-reactive antibody titers and intracellular Ca^{2+} levels. **B,** Lack of a significant association between anti-dsDNA antibody titers and intracellular Ca^{2+} levels. Values on the x-axis in **B** are the base 10 logarithm. Circles represent individual samples. See Figure 1 for definitions.

between anti-dsDNA antibody titer and cell viability in SLE (R^2 = 0.05, P = 0.46; n = 14) (Figure 1B). These results indicate that NR2-reactive antibody inhibits cell viability in a dose-dependent manner.

Effects of NR2-reactive antibody on NMDA receptor–related Ca^{2+} influx into cells. Because NMDA receptor is involved in the regulation of intracellular Ca^{2+} levels, we investigated the effect of NR2-reactive antibody on intracellular Ca^{2+} levels. NR1/NR2a-transfected HEK 293 cells were incubated with healthy control IgG (0.1 mg/ml; n = 9), NR2-reactive antibody–negative SLE IgG (0.1 mg/ml; n = 5), or NR2-reactive antibody–positive SLE IgG (0.1 mg/ml; n = 9). The HEK 293 cells in conditioned media were stimulated with 10 μM NMDA, and the intracellular Ca^{2+} levels were evaluated by Fluo-3 and confocal laser scanning microscopy. Data were normalized to the fluorescence intensity in cells exposed to 10 μM A23187. The value of NR2-reactive antibody is expressed as the OD value. Regression analysis was performed with NR2-reactive antibody as the independent variable and intracellular Ca^{2+} level as the dependent variable. We also investigated the association between anti-dsDNA antibody titer and intracellular Ca^{2+} level. Anti-dsDNA antibody was not detected in the 9 healthy controls.

There was a significant association between the NR2-reactive antibody titer and the intracellular Ca^{2+} level (R^2 = 0.69, $P < 0.0001$; n = 23) (Figure 2A). In contrast, there was no significant association between the anti-dsDNA antibody titer and the intracellular Ca^{2+} level in SLE (R^2 = 0.12, P = 0.22; n = 14) (Figure 2B). These results indicate that NR2-reactive antibody specifically increases the intracellular Ca^{2+} level in a dose-dependent manner.

Comparison of the intracellular Ca^{2+} level in NR1/NR2a-transfected HEK 293 cells incubated in the presence or absence of NR2-reactive antibody–positive IgG. Figure 3 shows the intracellular Ca^{2+} level in NR1/NR2a-transfected HEK 293 cells incubated with healthy control IgG (0.1 mg/ml; n = 9), NR2-reactive antibody–negative SLE IgG (0.1 mg/ml; n = 5), or NR2-reactive antibody–positive SLE IgG (0.1 mg/ml; n = 9). NR1/NR2a-transfected HEK 293 cells treated with the various IgG fractions were exposed to the indicated concentrations of NMDA.

Figure 3A shows the intracellular Ca^{2+} level as determined by confocal laser scanning microscopy in unstimulated cells and after the addition of 10 μM or 100 μM NMDA or 10 μM A23187 to each subset. Figure 3B shows that as the concentration of NMDA increased, the level of intracellular free Ca^{2+} increased in NR1/NR2a-transfected HEK 293 cells treated with each IgG. The intracellular Ca^{2+} level was significantly higher in NR1/NR2a-transfected HEK 293 cells treated with NR2-reactive antibody–positive SLE IgG than in those treated with healthy control IgG or NR2-reactive antibody–negative SLE IgG in the presence of 10 μM NMDA ($P < 0.0001$ and P = 0.0002, respectively) (Figures 3A and B). The intracellular Ca^{2+} level was higher in NR1/NR2a-transfected HEK 293 cells treated with NR2-reactive antibody–positive SLE IgG than in those treated with the other 2 IgG samples in the presence of 100 μM or 1,000 μM NMDA, although the differences were not statistically significant.

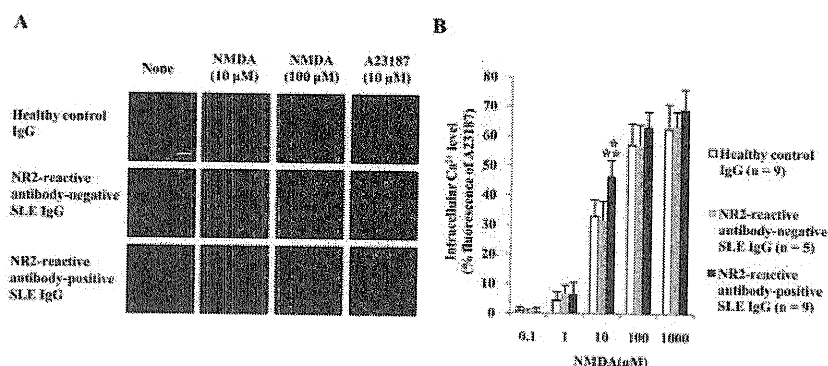

Figure 3. Comparison of intracellular Ca^{2+} levels in HEK 293 cells transfected with *N*-methyl-D-aspartate (NMDA) receptor subunits NR1/NR2a and incubated with and without NR2-reactive antibody. **A**, Intracellular Ca^{2+} levels as determined by confocal laser scanning microscopy in the absence of stimulation and in the presence of 10 μM or 100 μM NMDA or 10 μM A23187. Representative results are shown. Bar = 100 μm. **B**, Comparison of intracellular Ca^{2+} levels in each subset. As the NMDA concentration increased, the level of intracellular free Ca^{2+} increased in NR1/NR2a-transfected HEK 293 cells treated with each IgG. The intracellular Ca^{2+} level was significantly higher in NR1/NR2a-transfected HEK 293 cells treated with IgG from NR2-reactive antibody–positive systemic lupus erythematosus (SLE) patients than in those treated with healthy control IgG or IgG from NR2-reactive antibody–negative SLE patients in the presence of 10 μM NMDA. Bars show the mean ± SEM. $* = P < 0.0001$ versus healthy control IgG; $** = P = 0.0002$ versus NR2-reactive antibody–negative SLE IgG. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

Effects of zinc on cell viability. Amino acid sequencing of NR2 showed that the receptor had a zinc-binding site, and that the binding contributed to the regulation of Ca^{2+} influx through NMDA receptor signaling. Moreover, NR2-reactive antibody detected in patients with SLE recognizes the amino acids that include the zinc-binding site. These findings prompted us to investigate the effects of zinc on the viability of NR1/NR2a-transfected HEK 293 cells exposed to NR2-reactive antibody. NR1/NR2a-transfected cells were cultured in DMEM containing zinc at 1 μM, 10 μM, or 100 μM. Cell viability was calculated as the percentage above the value obtained in control cells (transfected with empty vector). As shown in Figure 4, as the concentration of zinc increased, greater cell viability was recovered in NR1/NR2a-transfected HEK 293 cells. However, cell viability was significantly lower in NR1/NR2a-transfected HEK 293 cells treated with NR2-reactive antibody–positive SLE IgG than in those treated with healthy control IgG or NR2-reactive antibody–negative SLE IgG in the presence of 10 μM zinc ($P < 0.0001$ for each comparison). These findings indicate that NR2-reactive antibody interacts with the zinc-binding site, resulting in a decrease in the protection of cell viability provided by zinc to NR1/NR2a-transfected HEK 293 cells.

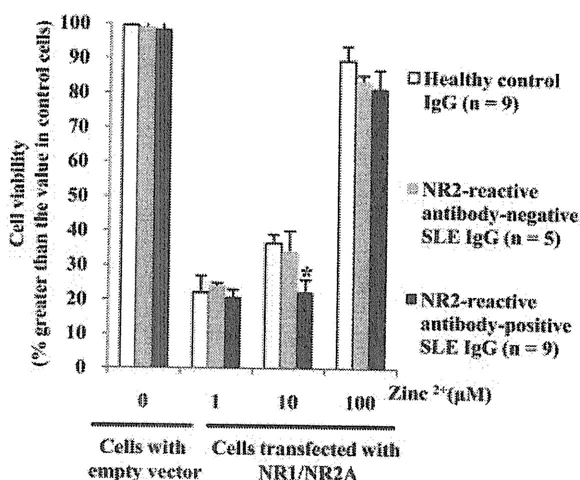

Figure 4. Effect of zinc on *N*-methyl-D-aspartate receptor subunit NR1/NR2a–transfected HEK 293 cell viability in the presence of IgG from each subset. As the concentration of zinc increased, the cell viability in NR1/NR2a-transfected HEK 293 cells treated with IgG from each subset increased. Cell viability was significantly lower in NR1/NR2a-transfected HEK 293 cells treated with IgG from NR2-reactive antibody–positive systemic lupus erythematosus (SLE) patients than in those treated with healthy control IgG or IgG from NR2-reactive antibody–negative SLE patients in the presence of 10 μM zinc. Bars show the mean ± SEM. $* = P < 0.0001$ versus healthy control IgG and NR2-reactive antibody–negative SLE IgG.

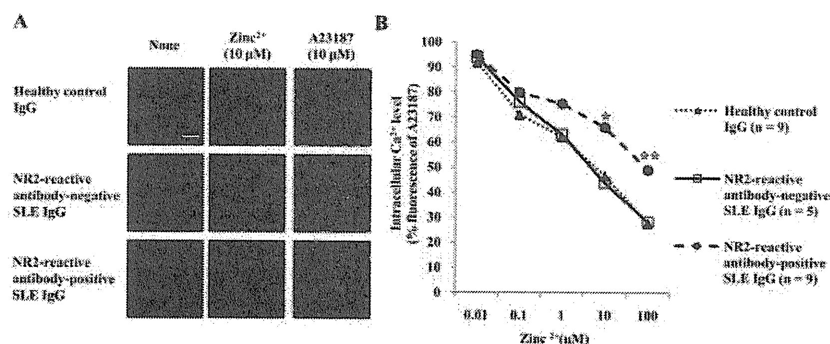

Figure 5. Efficacy of zinc as a modulator of NMDA receptor–induced intracellular Ca^{2+} level. **A,** Intracellular Ca^{2+} levels as determined by confocal laser scanning microscopy in the absence of stimulation and in the presence of both 10 μM NMDA and 10 μM zinc or in the presence of 10 μM A23187 alone. Representative results are shown. Bar = 100 μm. **B,** Efficacy of zinc as a modulator of intracellular Ca^{2+} level in the presence or absence of NR2-reactive antibody. NR1/NR2a-transfected HEK 293 cells treated with IgG from each subset were exposed to 10 μM NMDA, followed by exposure to zinc at 0.01 μM, 0.1 μM, 1.0 μM, 10 μM, or 100 μM. As the concentration of zinc increased, the level of intracellular free Ca^{2+} decreased in NR1/NR2a-transfected HEK 293 cells treated with IgG from each subset. Intracellular Ca^{2+} was higher in NR1/NR2a-transfected HEK 293 cells treated with NR2-reactive antibody–positive SLE IgG than in those treated with healthy control IgG or NR2-reactive antibody–negative SLE IgG in the presence of 10 μM zinc and in the presence of 100 μM zinc. Values are the mean. * = $P = 0.013$ versus healthy control IgG and $P = 0.0157$ versus NR2-reactive antibody–negative SLE IgG; ** = $P = 0.019$ versus healthy control IgG and $P = 0.047$ versus NR2-reactive antibody–negative SLE IgG. See Figure 3 for definitions.

Efficacy of zinc as a modulator of NMDA receptor–stimulated intracellular Ca^{2+} levels. Figure 5 shows the efficacy of zinc as a modulator of the intracellular Ca^{2+} level related to NMDA activity in the presence or absence of NR2-reactive antibody. NR1/NR2a-transfected HEK 293 cells treated with each IgG were exposed to 0.01 μM, 0.1 μM, 1 μM, 10 μM, or 100 μM zinc. The intracellular Ca^{2+} level was evaluated as described above and is expressed as the mean ± SEM.

Figure 5A shows the intracellular Ca^{2+} levels determined by confocal laser scanning microscopy when each subset of cells was exposed to media alone, to both 10 μM NMDA and 10 μM zinc, or to 10 μM A23187 alone. Figure 5B indicates that as the concentration of zinc increased, the levels of intracellular Ca^{2+} decreased in NR1/NR2a-transfected HEK 293 cells treated with IgG from each subset. After exposure to 10 μM zinc, the intracellular Ca^{2+} levels were significantly higher in NR1/NR2a-transfected HEK 293 cells treated with NR2-reactive antibody–positive SLE IgG than in those treated with healthy control IgG or NR2-reactive antibody–negative SLE IgG ($P = 0.013$ and $P = 0.0157$, respectively) (Figure 5B). This finding indicates that NR2-reactive antibody–positive SLE IgG weakens the efficacy of zinc as a negative modulator of intracellular Ca^{2+} levels. When 100 μM zinc was used, the concentrations of intracellular Ca^{2+} were significantly higher in NR1/NR2a-transfected HEK 293 cells treated with NR2-reactive antibody–positive SLE IgG than in those treated with healthy control IgG or NR2-reactive antibody–negative SLE IgG ($P = 0.019$ and $P = 0.047$, respectively).

DISCUSSION

We have demonstrated that NR2-reactive antibody derived from SLE patients inhibits cell viability through Ca^{2+} influx. DeGiorgio and colleagues demonstrated that NR2-reactive antibody mediates neuronal death via an apoptotic pathway in vitro and in vivo (9). Kowal et al reported that NR2-reactive antibody causes cognitive impairment when it accesses the CNS through a breach in the blood–brain barrier (17). Faust and colleagues have demonstrated that NR2-reactive antibody stimulates NMDA receptor–mediated synaptic responses and excitotoxicity through enhanced mitochondrial permeability. In the present study, we did not determine whether NR2-reactive antibody also reacts with NR2b. However, Faust and colleagues have shown

that human SLE autoantibodies bind both NR2a and NR2b (18).

NR2a-containing NMDA receptor and NR2b-containing NMDA receptor play different roles both in vitro and in vivo (27). In mature cortical cultures, activation of NR2a-containing NMDA receptor promotes neuronal survival and exerts a neuroprotective action against neuronal damage. In contrast, activation of NR2b-containing NMDA receptor results in excitotoxicity, increasing neuronal apoptosis (27). These findings may indicate that human SLE autoantibodies cause neuronal damage through inhibition of NR2a-containing NMDA receptor signaling or activation of NR2b-containing NMDA receptor signaling. On the other hand, overstimulation of NR2 can cause excitotoxic neuronal death through excessive entry of Ca^{2+} into cells (9,28–30).

Although the appropriate intracellular Ca^{2+} level is important for cell survival, an excessive Ca^{2+} load can trigger different cell death programs, such as activation of protease, caspase, and other catabolic processes (31). Intracellular Ca^{2+} is stored in the endoplasmic reticulum or mitochondria. The intracellular Ca^{2+} level in unstimulated cells is maintained at <100 n*M* by both uptake into the endoplasmic reticulum and extrusion into the extracellular space by the plasma membrane Ca^{2+} ATPase (32). Excessive Ca^{2+} influx into cells triggered by several agents (e.g., Ca^{2+} ionophores) promotes opening of the mitochondrial permeability transition pore, resulting in the release of cytochrome c and other proapoptotic proteins from mitochondria and the induction of apoptosis (32). In the present study, NR2-reactive antibody titer was correlated with intracellular Ca^{2+} level. Our results are consistent with those reported by Faust and colleagues (18).

The ligands of NR1 and NR2 are glycine and glutamate, respectively. NMDA receptor is composed of glycine-binding NR1 and glutamate-binding NR2 subunits. The binding of both glycine and glutamate activates intracellular Ca^{2+} signaling (1,19). In addition, the gating of NR2 is controlled by the region formed by the NR2 amino-terminal domain and the linker connecting the NR2 amino-terminal domain to the NR2 agonist-binding domain (33).

Zinc is an allosteric inhibitor of NMDA receptor. Allosteric inhibitors likely are critical in the regulation of NMDA receptor activity (33–35). In the present study, it was clear that higher concentrations of zinc inhibited intracellular Ca^{2+} influx in NR1/NR2a-transfected HEK 293 cells through the addition of NMDA. We also showed that the efficacy of zinc as a negative modulator of intracellular Ca^{2+} influx was significantly weaker in HEK 293 cells treated with NR2-reactive antibody–positive IgG than in those treated with NR2-reactive antibody–negative IgG. In addition, the viability of HEK 293 cells significantly decreased in NR2-reactive antibody–positive IgG compared to NR2-reactive antibody–negative IgG. These findings indicate that NR2-reactive antibody decreases the efficacy of zinc in regulating NMDA receptor activity. The NR2 amino-terminal domain sequence around Asp^{283} is important because zinc binding to Asp^{283} modulates Ca^{2+} signaling in cells expressing NR2a/NR2b. Our observations suggest that NR2-reactive antibody blocks the zinc-binding site, promotes intercellular Ca^{2+} influx, and induces apoptosis. Unexpectedly, anti-NR antibody did not directly stimulate the NMDA receptor (NR1 or NR2) to increase Ca^{2+} influx, suggesting that NR2-reactive antibody in SLE patients is neither agonistic nor antagonistic.

Some anti-dsDNA antibodies cross-react with NR2 and damage neuronal cells via an apoptotic pathway (9). Not all anti-dsDNA antibodies cross-react with NR2 to the same degree. The frequency of serum NR2-reactive antibody positivity is ~30–40% in patients with SLE (10–14). In the present study, we found no statistically significant correlation between NR2-reactive antibody titers and anti-dsDNA antibody titers. Kowal and colleagues showed that NR2-reactive antibody that reaches the CNS through the blood–brain barrier may potently damage neuronal cells (17). Two main mechanisms for blood–brain barrier damage in SLE have been described: microthrombi in cerebral vessels and immune-mediated attack of the endothelium (36). Anti-NR1/NR2 antibody has been detected in paraneoplastic encephalitis associated with ovarian teratoma and is believed to be produced to cross-react with teratoma as an antigen (6). Both the induction of NR2-reactive antibody in peripheral organs and the impairment of the blood–brain barrier could lead to neuropsychiatric syndrome in SLE patients. In NR2-reactive antibody–associated NPSLE, treatments should be considered that not only eliminate NR2-reactive antibody, but also protect the integrity of the blood–brain barrier and increase the zinc concentration in the CNS.

In conclusion, NR2-reactive antibody has a unique function that binds to the zinc-binding site of NR2 and that inhibits the biologic effects of zinc. NR2-reactive antibody decreases cell viability by Ca^{2+} influx in SLE.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Kawaguchi had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Gono, Kawaguchi, Yoneda, Yamanaka.
Acquisition of data. Gono, Takarada, Fukumori, Kaneko, Hanaoka.
Analysis and interpretation of data. Gono, Takarada, Fukumori, Kawaguchi, Katsumata.

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Anti-C1q Antibodies Are Associated With Systemic Lupus Erythematosus Global Activity but Not Specifically With Nephritis

A Controlled Study of 126 Consecutive Patients

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Objective. Several studies have shown that anti-C1q antibodies correlate with the occurrence and activity of nephritis in systemic lupus erythematosus (SLE). However, the significance of anti-C1q antibodies in SLE has not been fully characterized. The aim of this study was to investigate associations between anti-C1q antibodies and clinical and serologic parameters of SLE.

Methods. An enzyme-linked immunosorbent assay kit was used to measure anti-C1q antibodies in the sera of 126 consecutive patients with active SLE who were admitted to our university hospital from 2007 through 2009. Sera obtained from patients with high titers of anti-C1q antibodies at the initial evaluation (n = 20) were reevaluated following treatment. Control sera were obtained from patients with other autoimmune diseases and from normal healthy control subjects (n = 20 in each group). Associations between anti-C1q antibodies and clinical and serologic parameters of SLE were statistically analyzed.

Results. Anti-C1q antibodies were detected in the sera of 79 of 126 patients with SLE. The prevalence and titers of anti-C1q antibodies were significantly ($P < 0.0001$) higher in SLE patients than in patients with rheumatoid arthritis, patients with systemic sclerosis, and normal healthy control subjects. The prevalence and titers of anti-C1q antibodies were not significantly associated with active lupus nephritis ($P = 0.462$ and $P = 0.366$, respectively). Anti-C1q antibody titers were significantly correlated with SLE Disease Activity Index 2000 scores and the levels of anti–double-stranded DNA antibodies, C3, C4, CH50, and C1q ($P < 0.0001$ for all comparisons). Moreover, anti-C1q antibody titers significantly decreased as clinical disease was ameliorated following treatment ($P = 0.00097$).

Conclusion. These findings indicate that anti-C1q antibodies are associated with SLE global activity but not specifically with active lupus nephritis.

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Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the presence of autoantibodies. The diagnosis of SLE can be difficult, because SLE is a great imitator of other diseases (1). Autoantibodies are clearly central to the pathogenesis of SLE, with different autoantibodies associated with different clinical features (2). Among these autoantibodies (>100), several have been associated with disease activity (1). Although anti–double-stranded DNA (anti-dsDNA) antibodies are the most extensively studied autoantibodies in SLE, others play a role in clinical manifestations such as autoimmune hemolytic anemia, thrombocytopenia, skin disease, and neonatal lupus (3).

C1q is a complex molecule consisting of a collagenous portion with globular heads, morphologically resembling a bundle of tulips. C1q is the first component of the classical pathway of complement activation, and its main function is to clear immune complexes (ICs)

from tissues and self antigens generated during apoptosis (4). The hereditary deficiency of this component is a known risk factor for the development of SLE (4).

Anti-C1q antibodies were first described by Uwatoko et al in 1984 (5). Whereas complexed IgG mainly binds to the globular portion of C1q, anti-C1q antibodies bind to the collagenous portion, which apparently is the main immunogenic region of the molecule and its Fab fragments (6). Initially, anti-C1q antibodies were observed in patients with hypocomplementemic urticarial vasculitis syndrome and in patients with SLE (7). Anti-C1q antibodies were subsequently reported to be present in various other diseases (8). It has also been reported that anti-C1q antibodies correlate with the occurrence and activity of lupus nephritis, especially proliferative lupus nephritis (7,9,10). Many clinical studies showed a high negative predictive value (NPV) of anti-C1q antibodies for the occurrence of (proliferative) lupus nephritis, ranging up to 100% (9,10). A pathogenic role for these antibodies in the development of lupus nephritis has been suggested (11). One study also showed that the prevalence of organ manifestations other than nephritis was the same in patients with and those without high titers of anti-C1q antibodies (12).

The actual occurrence of anti-C1q antibodies in patients with active lupus nephritis remains controversial (9). Gunnarsson et al observed anti-C1q antibodies in only 11 of 18 patients with proliferative lupus nephritis (13). Heidenreich et al reported that tests for anti-C1q antibodies and ICs performed worse than tests for anti-dsDNA antibodies for the differentiation of lupus nephritis from other forms of glomerulonephritis (14). In their randomized controlled trial for proliferative lupus nephritis, Grootscholten et al showed that the prevalence of anti-C1q antibodies was 65%, and that renal flares were not preceded by increases in the titer of anti-C1q antibodies (15). Marto et al did not observe differences in the prevalence or levels of anti-C1q antibodies when comparing proliferative and nonproliferative forms of nephritis and suggested that results of other studies of a small number of patients—particularly those with nonproliferative nephritis—do not hold true in a large patient cohort (4). Most previous studies were retrospective (patients were not consecutively or randomly sampled) (4,10,12,16) and frequently were small in size (9,17). Thus, the significance of anti-C1q antibodies in SLE and lupus nephritis has not been fully characterized, and testing for these antibodies still does not have a defined place in routine clinical practice (4). The aim of the current study was to investigate associations between anti-C1q antibodies and clinical and serologic parameters of SLE in a larger controlled study of consecutive patients.

PATIENTS AND METHODS

Patients. We performed a case–control study of patients who were treated for active SLE at the Tokyo Women's Medical University hospital from 2007 through 2009. A total of 126 consecutive patients with active SLE were identified using the Tokyo Women's Medical University SLE Database, and sera were obtained from these patients. All of these patients fulfilled at least 4 of the American College of Rheumatology (ACR) revised criteria for SLE (18,19). At our institution, patients suspected of having SLE or those with newly diagnosed SLE were typically admitted for systemic evaluation regardless of the severity of disease and were eligible for inclusion in the study. Patients in whom SLE was previously diagnosed and who experienced disease flares were also included. Of the 135 patients admitted during the study period, 9 were excluded from the study because of the unavailability of samples or the lack of informed consent.

At the time of admission to the hospital, each patient completed a standardized medical history, including medication use, and was given a physical examination. Serology profiling for each patient was performed using the standard immunoassays described below. Serum samples that were obtained prospectively for another study were acquired before initiation or reinforcement of treatment, either at the time of admission or at the time of renal biopsy (when performed), and stored at −80°C. Treatment with corticosteroids or immunosuppressive drugs was instituted following completion of these evaluations. Sera from patients with high titers of anti-C1q antibodies (>125 units/ml) at the initial evaluation were reevaluated after disease was ameliorated by treatment (n = 20); these reevaluation samples were collected cross-sectionally in April or May, 2010. The mean ± SD interval between the time of initial evaluation and reevaluation was 645 ± 342 days. Control sera were obtained from age- and sex-matched normal healthy control subjects and from patients with rheumatoid arthritis (RA) or systemic sclerosis (SSc), diagnosed using standard criteria (20,21). This study was approved by the ethics committee of our institution, and the principles of the Helsinki Declaration were followed throughout the study. Informed consent was obtained from all participants.

Data collection. The information obtained from the medical records of the patients included demographic data such as age at the time of initiation of treatment, the clinical manifestations of SLE, and laboratory values. SLE disease activity was measured using the SLE Disease Activity Index 2000 (SLEDAI-2K) (22). Each SLE-related feature was defined according to the revised ACR criteria for SLE (18,19) or, if the feature was not included in the criteria, the SLEDAI-2K (22). For example, active nephritis was defined as persistent proteinuria of >0.5 gm/day (or >3+ if quantification was not performed) or by the presence of cellular casts in urine. Leukopenia was defined as a decrease in the number of white blood cells to $<4,000/mm^3$. Central nervous system (CNS) lupus was defined and diagnosed according to the standardized

ACR nomenclature and case definitions for neuropsychiatric lupus syndromes (23). We included only 12 CNS syndromes in the present study because of the substantial differences in anatomy, function, and clinical characteristics between the central and peripheral nervous systems (24,25). Anemia was defined as a decrease in the concentration of hemoglobin to <10.0 gm/dl due to any cause. Urticarial vasculitis was defined both clinically, as persistent urticarial skin lesions, and microscopically, as leukocytoclastic vasculitis (26). In some cases, renal biopsies were performed, and the histologic findings were classified according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) criteria (27).

Measurement of complement and anti-dsDNA antibodies. Levels of C3 and C4 were measured by turbidimetric immunoassay. CH50 was measured using a modification of the method of Mayer. Levels of serum ICs were measured by a C1q solid-phase enzyme immunoassay. Levels of serum C1q complement component were measured by rate nephelometry. Levels of serum anti-dsDNA antibodies were measured using the Farr radioimmunoassay.

Enzyme-linked immunosorbent assay (ELISA) for anti-C1q antibodies. Anti-C1q antibodies were measured using a solid-phase ELISA kit (Bulhmann Laboratories) according to the manufacturer's protocol. Briefly, calibrators, control sera, and patient sera (stored samples) were diluted in high salt buffer (0.5*M* NaCl) to avoid false-positive results by binding of ICs (28) and were incubated with human C1q adsorbed onto microtiter wells. After washing, horseradish peroxidase–labeled anti-human IgG conjugate was added, followed by a second washing step and the addition of tetramethylbenzidine substrate. The intensity of the blue color developed was in proportion to the amount of anti-C1q antibodies bound in the initial step. The reaction was terminated by the addition of 0.25*M* H_2SO_4. The absorbance was measured in a microtiter plate reader (Bio-Rad) at a wavelength of 450 nm and converted into units (units/ml) by plotting against the autoantibody titer of the calibrators/standards given by the manufacturer. The assay range was 1.0–400 units/ml. The cutoff value suggested by the manufacturer (15 units/ml) was obtained by testing the samples from 220 normal healthy blood donors using the same assay procedure. In some analyses in the present study, an increased cutoff value of 40 units/ml, as proposed by Trendelenburg et al, was used in order to achieve results comparable with those of previous studies (9). Sera from 20 patients with high titers of anti-C1q antibodies (>125 units/ml) at the initial evaluation were reevaluated when disease improved with treatment. Sera from patients with RA, patients with SSc, and normal healthy control subjects were also tested (n = 20 in each control group).

Statistical analysis. Associations between anti-C1q antibodies and clinical and serologic parameters of SLE were analyzed using Fisher's exact test for categorical variables and the Mann-Whitney U test for continuous variables for 2-group comparisons. Comparisons of 3 or more groups were performed using the Kruskal-Wallis test, and Steel's multiple comparison test for continuous variables and the chi-square test for categorical variables. The relationships between anti-C1q antibody levels and other continuous variables were analyzed using Spearman's rank correlation. The anti-C1q antibody levels before and after treatment were compared using Wilcoxon's signed rank test. *P* values less than 0.05 were considered significant. All tests were 2-tailed. The sensitivity, specificity, positive predictive value (PPV), and NPV of anti-C1q antibodies were also calculated. All analyses were performed using JMP statistical software (SAS Institute).

RESULTS

Demographic characteristics of the patients with SLE. Of the 126 patients with SLE enrolled in the present study, 123 were women, and 3 were men. The patients ranged in age from 17 years to 77 years (median age 37 years). With the exception of 2 Chinese women, all of the patients were Japanese. Twenty-one patients had clinically active nephritis. Renal biopsies were performed to confirm lupus nephritis by histopathology in 20 of these patients. Because of complications, a renal biopsy was not performed in 1 patient. Of the 20 patients who underwent biopsy, 1 patient had class I, 1 patient had class II, 3 patients had class III, 5 patients had class IV, and 10 patients had class V lupus nephritis, according to the abbreviated version of the ISN/RPS classification system (27); combined classes III/V and IV/V were considered as class III and IV, respectively. Contrary to previous reports, the prevalence and titers of anti-dsDNA antibodies in these 21 patients were not significantly associated with active lupus nephritis ($P = 0.974$ and $P = 0.628$, respectively).

Higher prevalence and titers of anti-C1q antibodies in patients with active SLE compared with patients with RA, patients with SSc, and normal healthy control subjects. Using the cutoff value of 15 units/ml, as recommended by the manufacturer of the ELISA kit, the prevalence of anti-C1q antibodies in patients with active SLE (79 of 126) was significantly higher than that in normal health control subjects (2 of 20), patients with RA (2 of 20), and patients with SSc (3 of 20) ($P < 0.0001$ for all). When samples from patients with SLE and normal healthy control subjects were compared, the sensitivity, specificity, PPV, and NPV for the diagnosis of SLE were 63%, 90%, 98%, and 28%, respectively. Using the higher cutoff value described by Trendelenburg et al (40 units/ml) (9), the prevalence of anti-C1q antibodies in patients with SLE (51 of 126) remained significantly higher than that in normal healthy control subjects (1 of 20), patients with RA (1 of 20), and patients with SSc (2 of 20) ($P = 0.002$, $P = 0.002$, and $P = 0.011$, respectively). Titers for anti-C1q antibodies were significantly higher in patients with SLE than in normal healthy control subjects or patients with RA or patients with SSc (the median values were 21.9 units/ml,

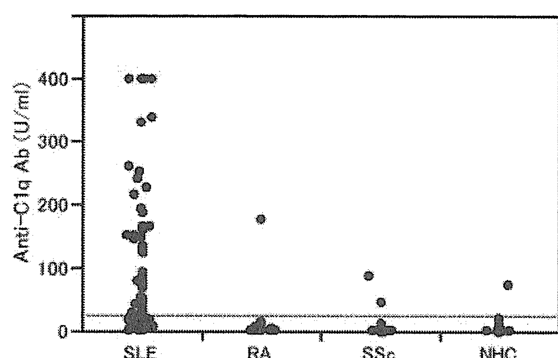

Figure 1. Anti-C1q antibody (Ab) levels in sera obtained from 126 consecutive patients with active systemic lupus erythematosus (SLE), 20 control patients with rheumatoid arthritis (RA), 20 control patients with systemic sclerosis (SSc), and 20 normal healthy controls (NHCs), as measured using an enzyme-linked immunosorbent assay (ELISA) kit. The broken line indicates the cutoff value (15 units/ml) recommended by the manufacturer of the ELISA kit. The titers of anti-C1q antibodies were significantly higher in patients with SLE than in normal healthy controls, patients with RA, and patients with SSc ($P< 0.0001$ for each comparison).

2.9 units/ml, 4.4 units/ml, and 2.5 units/ml, respectively; $P < 0.0001$ for all) (Figure 1).

Associations between SLE-related clinical features and prevalence and titers of anti-C1q antibodies. Anti-C1q antibodies were detected in the sera of patients showing various clinical manifestations of active SLE (Table 1). The prevalence of anti-C1q antibodies was significantly higher in SLE patients with leukopenia ($P = 0.029$) or hypocomplementemia ($P < 0.0001$) and in those who were positive for anti-dsDNA antibodies ($P < 0.0001$) and ICs ($P < 0.0001$) than in those without these features. Contrary to previous reports, the prevalence of anti-C1q antibodies was not significantly associated with active nephritis ($P = 0.462$), although the NPV of anti-C1q antibodies for active nephritis was as high as 87%, in accordance with previous reports (7,9).

The titer of anti-C1q antibodies was significantly higher in SLE patients with leukopenia ($P = 0.046$), anemia ($P = 0.027$), or hypocomplementemia ($P < 0.0001$) and in those who were positive for anti-dsDNA antibodies ($P < 0.0001$) and ICs ($P < 0.0001$) than in those without these features (Table 2). Also in disagreement with previous reports, we observed that the titer of anti-C1q antibodies was not significantly associated with active nephritis ($P = 0.366$). Even when active nephritis was subcategorized into "proliferative lupus nephritis" (class III and IV according to the ISN/RPS criteria) (4) and other categories, we observed no significant difference in the prevalence of anti-C1q antibodies in patients with proliferative lupus nephritis and those without active nephritis (7 of 8 patients and 64 of 105 patients, respectively; $P = 0.257$). Interestingly, in the 2 SLE patients with urticarial vasculitis, the prevalence and titers of anti-C1q antibodies were very high

Table 1. Associations between systemic lupus erythematosus–related clinical features and incidence of anti-C1q antibodies*

	Anti-C1q antibody						
Clinical feature	Positive (n = 79)	Negative (n = 47)	*P*	Sensitivity, %	Specificity, %	PPV, %	NPV, %
Malar rash/discoid rash	31 (39)	13 (28)	0.247	70	41	39	72
Urticarial vasculitis	2 (3)	0 (0)	0.529	100	38	3	100
Alopecia	6 (8)	3 (6)	1.000	67	38	8	94
Oral or nasal ulcers	2 (3)	1 (2)	1.000	67	37	3	98
Serositis	5 (6)	3 (6)	1.000	63	37	6	94
Arthritis	24 (30)	10 (21)	0.305	71	40	30	79
Active nephritis	15 (19)	6 (13)	0.462	71	39	19	87
CNS lupus	3 (4)	2 (4)	1.000	60	37	4	96
Vasculitis	2 (3)	2 (4)	0.629	50	37	3	96
Myositis	2 (3)	0 (0)	0.529	100	38	3	100
Fever >38°C	15 (19)	7 (15)	0.634	68	38	19	85
Thrombocytopenia	7 (9)	3 (6)	0.743	70	38	9	94
Leukopenia	15 (19)	2 (4)	0.029	88	41	19	96
Anemia	21 (27)	7 (15)	0.183	75	41	27	85
Positive anti-dsDNA antibodies	62 (78)	14 (30)	<0.0001	82	66	78	70
Hypocomplementemia	62 (78)	20 (43)	<0.0001	76	61	78	57
Positive immune complex	38 (48)	1 (2)	<0.0001	97	53	48	98
Positive ANAs	65 (94)	35 (83)	0.076	65	62	94	17

* Values are the number (%). *P* values were determined by Fisher's exact test. PPV = positive predictive value; NPV = negative predictive value; CNS = central nervous system; anti-dsDNA = anti-double-stranded DNA; ANAs = antinuclear antibodies.

Table 2. Associations between SLE-related clinical features and titers of anti-C1q antibodies

Feature	Present	Absent	P†
Malar rash/discoid rash	38.7	21.3	0.085
Urticarial vasculitis	152.7	21.8	0.160
Alopecia	18.1	22.2	0.751
Oral or nasal ulcers	400.0	21.9	0.313
Serositis	36.8	21.9	0.916
Arthritis	23.8	21.8	0.357
Active nephritis	29.8	21.8	0.366
CNS lupus	15.8	22.0	0.508
Vasculitis	13.6	22.0	0.310
Myositis	22.6	21.9	0.907
Fever >38°C	49.3	21.7	0.175
Thrombocytopenia	85.9	21.9	0.189
Leukopenia	48.1	20.8	0.046
Anemia	46.6	20.8	0.027
Positive anti-dsDNA antibodies	54.2	9.9	<0.0001
Hypocomplementemia	39.9	9.9	<0.0001
Positive immune complex	165.7	15.8	<0.0001
Positive ANAs	23.8	13.4	0.077

* Values are the median units/ml of anti-C1q antibodies in patients with or without the clinical feature of systemic lupus erythematosus (SLE). CNS = central nervous system; anti-dsDNA = anti-double-stranded DNA; ANAs = antinuclear antibodies.
† By Mann-Whitney U test.

(Tables 1 and 2, respectively), although the number of these patients was too small to reach statistical significance.

Correlations between anti-C1q antibody titers and markers of disease activity. When assessing the correlation between anti-C1q antibody titers and an established index and known serologic markers of disease activity, the titers of anti-C1q antibodies were correlated with the SLEDAI-2K, anti-dsDNA antibodies, C3, C4, CH50, and IC by C1q assay (Figures 2A, B, C, D, E, and F, respectively). For all comparisons, there were significant positive or negative correlations (all $P < 0.0001$).

Correlation between anti-C1q antibody titers and levels of C1q. When anti-C1q antibody titers were compared with the levels of C1q in patients with SLE, a significant negative correlation was observed ($P < 0.0001$), as shown in Figure 3. The fact that whether or not the level of C1q decreased beyond its normal range (8.8–15.2 mg/dl) in each patient was also significantly associated with the presence of anti-C1q positivity (>15 units/ml), as determined using Fisher's exact test for categorical variables ($P = 0.0010$).

Anti-C1q antibody titers before and after treatment in patients with SLE who had high titers of anti-C1q antibodies at the initial evaluation. In a subgroup of 20 patients with SLE who had high titers of anti-C1q antibodies (>125 units/ml) at the initial evaluation, retesting showed that anti-C1q antibody titers decreased significantly in accordance with disease amelioration following treatment (median decrease 84%

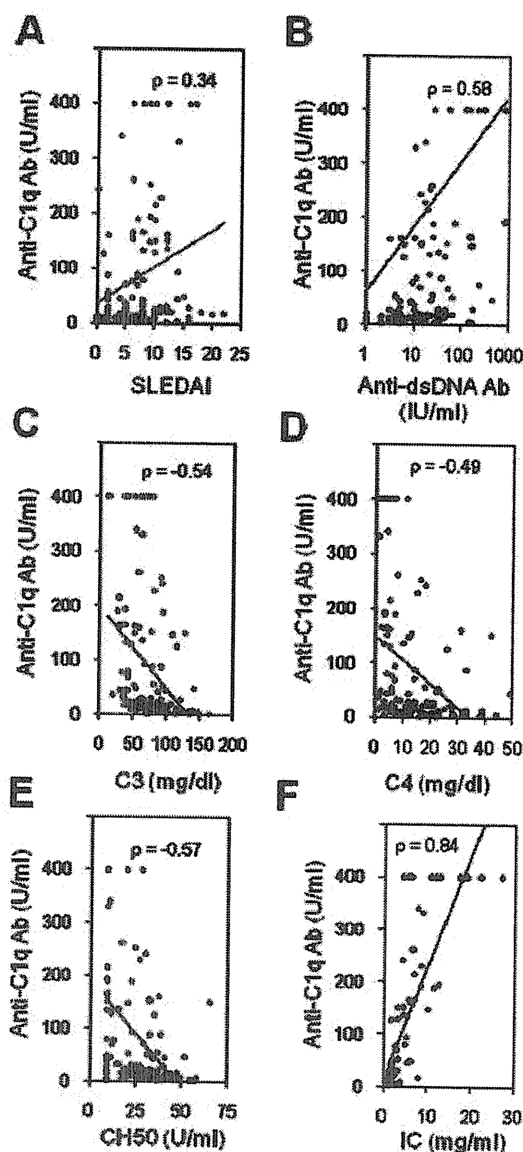

Figure 2. Correlations between anti-C1q antibody (Ab) titers and the Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI) score (**A**), anti–double-stranded DNA (anti-dsDNA) antibodies (**B**), C3 (**C**), C4 (**D**), CH50 (**E**), and immune complexes (ICs) (**F**), as measured in 126 consecutive patients with active SLE. For all comparisons, significant positive or negative correlations ($P < 0.0001$) were observed.