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Extended Report

Autoantibodies to a NR2A peptide of the glutamate/NMDA receptor in sera of patients with systemic lupus erythematosus.

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Running title: Anti-NMDA receptor antibodies in SLE

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Abbreviations: AMPA, 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl); ANA, anti nuclear antibodies; APS I, autoimmune polyendocrine syndrome type I; CNS, central nervous system; dsDNA, double stranded deoxyribonucleic acid; MG, myasthenia gravis; NMDA, N-methyl-D-aspartic acid; SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index.
ABSTRACT

Objectives: To determine the prevalence of autoantibodies directed against an epitope of the glutamate/N-methyl-D-aspartic acid (NMDA) receptor subunit NR2A (which is highly expressed in human brain) in the sera of lupus patients, and to investigate the possible correlation of these antibodies with clinical and serological manifestations of systemic lupus erythematosus (SLE).

Methods: Sera were obtained from 109 consecutive SLE patients. As controls we tested sera of 65 patients with myasthenia gravis (MG), 19 patients with autoimmune polyendocrine syndrome type I (APS I) and 65 healthy donors. A 15 amino acid long peptide based on a sequence within the NR2A subunit of the NMDA/glutamate receptor was synthesized. Antibodies to this peptide was determined by ELISA. Antibodies against double-stranded DNA (dsDNA) were measured by Chrithidia Luciliae assay. Disease activity was determined using the SLE disease activity index (SLEDAI).

Results: Sera of 34/109 (31%) SLE patients reacted specifically with the NR2A peptide compared to only 4/65 (6.1%, p<0.001) MG patients, 1/19 (5.3%, p<0.02) APS I patients and 3/65 (4.6%, p<0.001) of healthy controls. No correlation was found between the presence of NR2A and dsDNA or anti-cardiolipin specific autoantibodies. In addition, no significant correlation was observed between the presence of NR2A specific antibodies and the SLEDAI score or any lupus-related clinical manifestations.

Conclusions: A significant number (31%) of SLE patients have NR2A specific antibodies that do not correlate with anti-dsDNA antibodies. Additional studies of lupus patients with neurological disorders should elucidate the role of NR2A specific antibodies in lupus-related CNS manifestations.
Key words: systemic lupus erythematosus, DNA-specific antibodies, NMDA glutamate receptor, central nervous system.
INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by impairment of B and T cell functions, cytokine dysregulation and immune complex depositions accompanied by systemic clinical manifestations [1,2]. The hallmark of SLE is the presence of a variety of autoantibodies directed mostly to double-stranded DNA (dsDNA), nuclear antigens, ribonucleoproteins and cell surface antigens [1-3]. Some lupus associated clinical manifestations appear to be related to or mediated by specific autoantibodies (e.g. anti dsDNA and lupus nephritis [4]; anti-cardiolipin antibodies and anti-phospholipid syndrome [5]). However, the mechanism(s) involved in the pathogenesis of SLE as well as the nature of the autoantigen(s) are not yet defined.

About 10% of lupus patients exhibit central nervous system (CNS) involvement, namely, psychosis or focal seizures [6,7]. Moreover, non-focal neuropsychiatric disturbances including headache and cognitive decline are observed in up to 50% of the patients during the course of their disease [7,8]. The mechanism(s) for these impairments is unknown, yet several studies have reported a correlation between lupus related neurological disorders and the presence of various autoantibodies directed against ribosomal P-protein [9], neurofilaments [10] or neuronal cells [11]. Recently, DiGiorgio et al [12] showed that a subset of anti-DNA antibodies (mouse monoclonal R4A antibodies and sera of some lupus patients) cross-react with a sequence within the glutamate/NMDA receptor subunit NR2. Furthermore, injection of the cerebrospinal fluid of a patient with SLE and progressive neurological decline which contained such cross-reactive anti-DNA antibodies into mouse brain, caused neuronal death [12]. These results suggested that cross-reactive antibodies which recognize a common pentapeptide Asp/Glu-Trp-Asp/Glu-Tyr-Ser/Gly present both in dsDNA and N-methyl-D-aspartic acid (NMDA) receptor subunits NR2A and NR2B, may mediate lupus related disorders of the central nervous system (CNS).
The NMDA receptors, which bind the neurotransmitter glutamate and are present on neuronal cells throughout the brain, were reported to play a role in many neurological functions including learning and memory [13]. Since other studies have reported the presence of NMDA receptors on non-neuronal cells, including platelets [14], autoantibodies directed against these receptors may also be involved in the pathogenesis of other (non-neurological) SLE related clinical manifestations.

The present study was conducted to determine the prevalence of antibodies against an epitope of the glutamate/NMDA receptor subunit NR2A in the sera of a large cohort of lupus patients, to define the correlation of the latter antibodies with dsDNA, specific antibodies and to investigate the possible correlation between the presence of the NR2A specific antibodies and SLE-related clinical manifestations and disease activity.

PATIENTS AND METHODS

Patients

One hundred and nine patients (16 men and 93 women) with SLE participated in the study. All patients revealed at least 4 of the American College of Rheumatology revised classification criteria for SLE [15]. The mean age at diagnosis was 34 ± 13.1 (mean ± SD) years and the mean follow up period was 14±10.8 (mean ± SD) years. All patients had anti-nuclear antibodies (ANA) in their sera (tested at 1:100 dilution) as determined by a Hep2 cell assay [16]. Eighty three percent of the patients had dsDNA specific reactivity as measured (at sera dilution of 1:10) by Chrithidia Luciliae assay [16] at least at one time point during the follow up period. The results of dsDNA reactivity are presented as dsDNA scores (negative, +1 to +4 scores). Anti-phospholipid antibodies determined by the presence of anti-cardiolipin antibodies and/or lupus anti-coagulants were detected in 28% of the patients. Arthritis was observed in 85/109 (78%) of patients. Hematological (hemolytic anemia and/or
thrombocytopenia and/or leukopenia and/or lymphopenia) and renal involvement were observed in 68% and 38% of the patients, respectively. Neurological manifestations, defined in the present study as psychosis or focal seizures were observed in 6 (5.5%) of the patients during the follow up period. Disease (SLE) activity was determined according to the SLEDAI lupus activity index [17]. The mean SLEDAI score at the time of the study was 6.32 ± 5.13 (range 0-25). Fifty percent of the patients were treated with steroids at the time of the study and 15% were treated with cytotoxic agents (either cyclophosphamide or azothropine or methotrexate). As controls we studied sera of 65 patients with myasthenia gravis (MG). Although MG is a neurological disease involving the neuromuscular junction rather than the brain, we chose MG patients as part of our controls because of the autoimmune nature of this disease. In addition, 19 patients with autoimmune polyendocrine syndrome type I (APS I) and 65 age and sex matched healthy controls were tested. All participants signed an informed consent form prior to the initiation of the study, which was approved by the ethical committee of the Kaplan Medical Center.

**Synthetic peptide**

The 15 amino acid peptide SVSYDDWDYSLEARV (amino acids 278-292) of the NR2A subunit of the NMDA subtype of the glutamate receptor that contains the DWDYS epitope [12], was synthesized with an automated synthesizer (Applied Biosystems model 430A) using the company’s protocol for t-butyloxycarbonyl strategy [18].

**Evaluation of antibodies against the NR2A peptide**

Antibodies against the NR2A peptide were measured by ELISA using 96-well microtiter plates (Nunc, Denmark). The plates were coated with 0.025µg peptide per well at 4°C over night. Plates were washed 4 times with phosphate-buffered saline containing 0.05% Tween-20
Blocking was performed with PBS containing 10% milk with 1% fat for 2 hours at room temperature. After washing with PBS-T, plates were incubated with sera dilutions (1:50) for 3 hours at room temperature. Following further washing, peroxidase-conjugated goat anti-human IgG was added at a 1:5000 dilution. Plates were developed using 2,2’azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (Sigma) and read after 30 min at 405 nm by an ELISA reader. In each assay a pool of 42 normal control sera were used as a routine standard negative control and a serum found in the initial ELISA to bind strongly to NR2A was used in all subsequent assays as a positive control. The positive and negative controls were tested in quadruplicate on each plate. The results were expressed as an NR2A index calculated as follows: (OD sample - OD negative control) / (OD positive control - OD negative control) x 100. The mean of the 65 normal controls + 2 SD was used as the upper limit of normal. The intra- and inter assay variability was 5 and 15%, respectively.

**Statistical analyses**

The results are presented as mean ± SD. The linear regression (R²) test (using Microsoft Excel), Chi-square and the Students t-tests were used for statistical analyses. P ≤ 0.05 was considered significant.

**RESULTS**

**NR2A-specific antibodies in sera of SLE patients.**

In order to assess the frequency of antibodies reactive with the NR2A-peptide in the sera of SLE patients, sera of 109 consecutive lupus patients, 65 patients with MG, 19 patients with APS I and 65 healthy donors were tested (at 1:50 dilution) in a NR2A specific ELISA. Figure 1 shows that 34/109 (31%) of lupus patients reacted specifically with the NR2A peptide.
compared to only 4/65 (6.1%, p<0.001) MG patients, 1/19 (5.3%, p<0.02) APS I patients and 3/65 (4.6%, p<0.001) of the healthy controls.

Relation between NR2A and dsDNA specific autoantibodies in sera of SLE patients

In order to investigate the possible relationship between NR2A and dsDNA-specific autoantibodies we further evaluated concomitantly the reactivity to NR2A and dsDNA in sera obtained from 59 of our SLE patients of whom 2 or more sera samples drawn at different time points were available for evaluation. As demonstrated in Figure 2, no correlation could be observed between dsDNA reactivity at different time points along the study and the presence and/or levels of NR2A specific antibodies. Moreover, sera with the highest reactivity of NR2A specific antibodies (NR2A index>80) were negative for dsDNA antibodies whereas most of the sera which exhibited high dsDNA reactivity (defined as dsDNA score of +4) did not react significantly with the NR2A peptide (Fig. 2, $R^2=0.037$). Analysis based on one serum sample of each patient also did not show any correlation ($R^2=0.0006$) between NR2A and dsDNA specific antibodies. In addition, there was no correlation ($R^2=0.0078$) between the levels of anti-phospholipid (anti-cardiolipin and/or lupus anti-coagulant) antibodies and NR2A specific antibodies. Similarly, the levels of ANA measured on Hep2 cells, did not correlate with the magnitude of NR2A activity ($R^2 = 0.0008$). The relationships between NR2A and dsDNA specific antibodies were further evaluated in 3 individual patients at different time points (Fig. 3). As can be seen in Figure 3A sera of patient number 1 did not react significantly with the NR2A peptide but had high titers (+4 score) of dsDNA specific antibodies at all time points during a 2.5 years follow up period. Patient number 2 also had no NR2A specific antibody reactivity in his sera whereas the anti-dsDNA antibody levels varied (between score 0 to +4) during the 18 months of the follow up (Fig. 3B). Finally, patient
number 3 had high levels of NR2A-specific antibodies, but no dsDNA autoantibodies during a follow up period of about 3 years (Fig. 3C).

**Relation between NR2A specific antibodies and clinical manifestations of SLE.**

All lupus related clinical manifestations (neurological, renal, hematological, mucucutaneous and musculoskeletal involvement) as well as lupus disease activity index (SLEDAI) did not correlate significantly \( (R^2 = 0.0238) \) with the presence and or titers of the NR2A antibodies in the sera of patients. The treatment with either steroids or cytotoxic drugs did not affect the presence or titers of the anti-NR2A antibodies.

It is noteworthy that the low number of patients (6/109, 5.5%) with neurological involvement defined as psychosis or seizure limited the ability to determine the possible association and the role of the NR2A antibodies in the development of lupus-related CNS disease. Although no significant correlation was observed between the presence of NR2A antibodies and lupus related CNS involvement, the two highest NR2A antibody indices were detected in sera obtained from patients with CNS manifestations. Furthermore, Figure 3C demonstrates high NR2A-specific antibody levels in the sera of a patient with severe neurological manifestations. In this particular SLE patient, who suffered from organic brain syndrome, cranial nerve disorders and visual disturbances, the titers of NR2A specific antibodies correlated with the severity of the neurological involvement. Thus, the clinical improvement of her neurological symptoms (July 2000) was associated with a significant decrease of the NR2A antibody index (Fig. 3C). When neurological manifestations worsened again (November 2002), the NR2A-antibody index also increased (Fig. 3C). During this period no anti-dsDNA antibodies were detected in serum samples of this patient. Nevertheless, further studies of SLE patients with well defined neuropsychiatric lupus manifestations (19) should determined the role of the NR2A specific antibodies in neurological lupus.
DISCUSSION

The main finding of the present study is that a significant number (34/109 = 31%) of SLE patients harbor in their sera elevated levels of antibodies directed against a peptide consisting of amino acids 278-292 of the NR2A-subunit of the NMDA subtype of glutamate receptors, i.e. anti-NR2A antibodies. The specific association of the NR2A antibodies with SLE is supported by the low frequency of these antibodies in sera of patients with myasthenia gravis (4/65), APS I (1/19) and healthy controls (3/65) shown in this study (Fig. 1).

Glutamate is the major excitatory neurotransmitter of the nervous system and plays a crucial role in a variety of key functions in the central nervous system, including learning and memory [13]. Glutamate has ionotropic (ion channel) receptors and the G-protein coupled metabotropic receptors [20]. The ionotropic glutamate receptors are further subdivided into the 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionic acid (AMPA)/kainate receptors and the NMDA receptors, based on their preferential affinities for the synthetic excitatory NMDA or AMPA, respectively [20,21]. Molecular cloning has revealed three receptor subunit families (NR1, NR2, and NR3A) which form hetero-oligomeric complexes in native NMDA receptor channels [21]. NMDA receptors are of major interest, as they are involved in many processes necessary for brain development including neuronal migration [20], patterning of afferent termination [21], and several forms of long-term synaptic plasticity [22]. In addition, NMDA receptors have also been implicated as a mediator of neuronal injury caused by excess glutamate (excitotoxicity) and taking place in many neurological disorders including stroke, epilepsy, brain trauma, dementia, and neurodegenerative disorders [23,24]. Thus, in principle, antibodies specific to the NMDA receptors may lead to neurological disturbances.
Antibodies against the NMDA receptor subunit families were demonstrated in sera of patients with non-autoimmune neurological disorders. Thus, antibodies to NR2B were recently found in sera of 13/15 patients with chronic forms of epilepsy [25]. Anti-NR2 (NR2A/2B) antibodies were reported also in sera of patients with ischemic stroke (94%), including transient ischemic attack (75%) [22]. We recently found anti-NR2A (amino acids 278-292) specific antibodies in ~20% of patients with different types of epilepsy (Ganor et al, unpublished data). DeGiorgio et al [12] demonstrated neuronal damage upon injection of anti-NR2 glutamate receptor binding antibodies of two sera and one CSF sample of SLE patients. We report here the presence of NR2A specific antibodies in 31% of the sera of a large cohort of SLE patients (Fig. 1).

The findings of the present study suggest that the anti-NR2A antibodies are distinct from anti-dsDNA autoantibodies, which are the hallmark of SLE. Although we have not performed absorption experiments, the latter suggestion is based on the lack of association between the titers of antibodies directed to dsDNA and to NR2A (Figs. 2,3). In fact, the results showed that patients with the highest dsDNA specific antibody titers, at the time of the test, had the lowest NR2A specific antibody indices and vice versa raising the possibility of a negative correlation between these antibodies.

It is noteworthy that DeGiorgio et al [12] reported that a mouse monoclonal (IgG2b, R4A) anti-DNA antibody and a limited number of human SLE sera with dsDNA-specific antibodies cross reacted with the NR2A and NR2B subunits of the NMDA receptor. It appears that the anti-NR2A antibodies found herein differ from the NR2 binding of the anti-dsDNA IgG2b R4A monoclonal antibody demonstrated in the latter study [12]. It is also likely that because of the different approaches of the two studies different subsets of NR2A-specific antibodies were investigated. Nevertheless, although we can not rule out cross reactivity between dsDNA and NR2A in a very limited number of sera, the NR2A specific antibodies that were
detected in the sera of a significant number of SLE patients studied herein (Fig. 1), did not correlate with anti-dsDNA reactivity (Figs. 2 and 3).

To conclude, a significant number (31%) of SLE patients have elevated levels of antibodies to the NR2A peptide of the glutamate / NMDA receptor. Our findings call for further studies of SLE patients with various neurological and cognitive manifestations (19) to explore the potential contribution of the NR2A specific antibodies to the lupus-related CNS involvement.

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REFERENCES


LEGENDS TO THE FIGURES

**Figure 1.** Antibodies against the NR2A peptide of the glutamate/NMDA receptor in sera of patients with SLE. Sera of 109 SLE patients, of 65 patients with MG, 19 patients with APS1 and 65 healthy controls were tested. NR2A specific antibodies were determined by ELISA. Results are expressed as NR2A antibody indices that were calculated as described in Patients and Methods. The horizontal line represents the upper normal limit (NR2A-antibody index of 30) defined as the mean of 65 normal controls + 2 SD. *p<0.001, **p<0.02.

**Figure 2.** Correlation between NR2A antibody indices and dsDNA reactivity in sera of SLE patients. The NR2A antibody indices and dsDNA antibody reactivity (negative to +4) were compared in 125 sera samples from 59 SLE patients (two or more sera samples per patient). The line represents the linear regression.

**Figure 3.** Correlation between NR2A antibody indices, dsDNA specific antibodies and disease activity (SLEDAI). NR2A antibody index, dsDNA reactivity and SLEDAI were determined in 3 different SLE patients (A,B,C) during a follow up period of 18 to 33 months.
$R^2 = 0.0367$
NR2A-Ab index

SLE       MG       APS I       Controls
n=109      n=65     n=19       n=65

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