Studies on Neuropsychiatric SLE

Steup-Beekman, G.M.

CHAPTER 4

Anti-NMDA receptor autoantibodies in patients with SLE and their first-degree relatives

G.M. Steup-Beekman[1], S.C.A. Steens[2], M.A. van Buchem[2] ,T.W.J. Huizinga[1]

Departments of [1]Rheumatology and [2]Radiology, Leiden University Medical Center, Leiden, the Netherlands

Lupus 2007;16(5):329-334

Abstract

Objective

The objective of this study is to investigate the presence of autoantibodies cross-reacting with the NR2 subunit of the *N*-methyl-D-aspartate (NMDA) glutamate receptor in plasma samples of patients with systemic lupus erythematosus (SLE), in their healthy first-degree relatives and in healthy unrelated individuals and to determine whether these autoantibodies are specific for lupus patients in general or for the subgroup of SLE patients with neuropsychiatric manifestations.

Methods

Plasma samples were collected from 51 lupus patients (19 with and 32 without neuropsychiatric manifestations), 161 first-degree relatives and 55 healthy unrelated controls. Antibodies to a linear peptide of the NR2 subunit of the NMDA receptor were determined by enzyme-linked immunosorbent assay.

Results

A significant difference in mean antibody reactivity between SLE patients and healthy unrelated controls ($P < 0.01$) and between first-degree relatives and healthy unrelated controls ($P < 0.001$) was found. No difference was found between lupus patients and their first-degree relatives or between lupus patients with and without neuropsychiatric symptoms.

Conclusion

In this study, anti-NMDA receptor autoantibodies show more specificity for lupus patients (but not for selected patients with neuropsychiatric symptoms) and their first-degree relatives than for healthy controls, indicating a familial basis to mount an immune response to this peptide.

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disorder and autoantibodies play a central role in the pathogenesis of the disease. In several studies, the presence of genetic susceptibility for autoantibody production in SLE was demonstrated; autoimmune diseases cluster within families of SLE patients and the prevalence of autoantibodies is raised in relatives of SLE patients.[1-3]

In neuropsychiatric manifestations of SLE, the exact role of autoantibodies remains controversial. Neuropsychiatric symptoms occur in up to 80% of all lupus patients, depending on patient selection and diagnostic criteria.[4,5] The wide range of neuropsychiatric manifestations varies from mild headache and subtle cognitive dysfunction to psychosis and transverse myelitis.[5] In 1999, the American College of Rheumatology (ACR) published case definition criteria for 19 neuropsychiatric syndromes in SLE in order to create more homogeneous patient groups to facilitate research.[6]

In 60% of the cases, neuropsychiatric disease can be attributed to lupus itself and is referred to as primary neuropsychiatric SLE (NPSLE).[5,7] In primary NPSLE, pathogenesis of diffuse neuropsychiatric syndromes remains largely unknown and conventional neuroimaging techniques often show non-specific abnormalities.[8] The presence of antiphospholipid (aPL) antibodies is correlated with focal syndromes such as stroke, but these antibodies might also be involved in diffuse neuropsychiatric manifestations such as cognitive dysfunction.[9,10] Besides aPL antibodies, intracranial angiopathy, cytokine and autoantibody-mediated neuronal dysfunction have been suggested as underlying processes in NPSLE. Autoantibodies can be directed against neural target cells and subsequently lead to non-lethal and reversible malfunction.[11,12] In 2001 a subset of anti-double stranded DNA (anti-dsDNA) antibodies cross-reacting with the NR2 subunit of the *N*-methyl-D-aspartate (NMDA) receptor was described.[13] This glutamate receptor is distributed in the whole brain and is important in processes that influence learning and memory.[14] Anti-NMDA receptor autoantibodies have been demonstrated in cerebrospinal fluid (CSF) of a patient with NPSLE and caused neuronal death when injected in mouse brain. In a subsequent study in which these antibodies were induced in mice, neuronal damage occurred only when a breach in the integrity of the blood-brain barrier was present.[15]

The aim of this study was to investigate the prevalence of autoantibodies against the NR2 subunit of the NMDA receptor in plasma samples of SLE patients, their first-degree relatives and non-related individuals to establish whether these antibodies are specific for SLE patients or for those lupus patients with neuropsychiatric manifestations.

Patients and Methods

Patients

Stored plasma samples were collected from 51 (48 females and 3 males) SLE patients, 161 disease-free first-degree relatives and 55 healthy unrelated controls. Samples were obtained in a study population described by van der Linden *et al*.[1]

Patients fulfilling at least four of the ACR 1982 revised criteria for the classification of SLE[16] from single case families who attended the Department of Rheumatology in the Leiden University Medical Center (LUMC) between January 1997 and October 1998, were enrolled. The LUMC is the only referral center for rheumatic patients in an area with 400.000 inhabitants. Overall disease activity was measured by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI).[17] All patients were evaluated clinically at study entry by taking history, physical examination and studying the charts. The medical records were reviewed with particular attention to documented neuropsychiatric manifestations present at any time from the date of SLE diagnosis. Neuropsychiatric involvement was considered to be present if formal neurological or psychiatric testing by consulting physicians proved to be abnormal and no other explanation for the neuropsychiatric manifestation could be found. In case of suspected cognitive dysfunction, neuropsychological testing was performed by an experienced neuropsychologist. The neuropsychiatric manifestations were classified retrospectively by two rheumatologists using the ACR nomenclature and case definitions for neuropsychiatric syndromes in SLE. [6] Nineteen SLE patients had a history of neuropsychiatric manifestations.

The first-degree relatives of SLE patients included parents, children and siblings. Healthy controls were unrelated subjects and consisted of 27 partners of the SLE patients and 28 partners and their family members of multiple sclerosis patients. All first-degree relatives and healthy controls were evaluated at study entry by a rheumatologist by taking history and physical examination. Of the first-degree relatives 2 subjects reported headache, but none of the first-degree relatives or healthy controls had any neurological impairment.

Plasma samples of each subject, prepared according to standard procedures (4 ml of citrated blood samples were centrifuged at 3000 g for 15 min), were stored at -70°C in the LUMC. The samples had been through a maximum of two freeze-thaw cycles prior to the current analysis. Of each subject, 50 µL of citrate plasma was collected and coded samples were sent to the group of B. Diamond in New York on dry ice for analysis.

All patients had given informed consent.

Anti-peptide (NR2) antibody testing

Immunoglobulin autoantibodies to a linear epitope (containing the pentapeptide sequence DWEYS) of the NR2 subunit of the NMDA receptor were assessed by enzyme-linked immunosorbent assay (ELISA) using 96-well microtitre plates.

On each plate five standard negative control sera were tested. The plates were read after 90 minutes. The anti-peptide antibody ELISA was performed as described previously by Putterman and Diamond.[18] Amidated, acetylated DWEYSVWLSN was synthesized at the peptide synthesis facility of the Albert Einstein College of Medicine, New York. Peptide purity was ≥ 90% as assayed by high-performance liquid chromatography. To assay for the presence of antipeptide antibodies, peptide at 20 µg/mL in phosphate-buffered saline (PBS) was adsorbed to Falcon Pro-Bind 96-well microtitre plates (Becton Dickinson, Lincoln Park, NJ, USA) at 4°C overnight. Plates were blocked with 3% fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT, USA) in PBS for 1 h at 37°C and then incubated with serum at a 1:500 dilution in PBS for 2 h at 37°C. The plates were then washed five times with PBS-Tween, and alkaline phosphatase-conjugated goat anti-mouse IgG or IgM (Southern Biotechnology Associates, Birmingham, AL, USA) diluted 1:1,000 in 3% FCS/PBS was added for 1 h at 37°C, followed by *p*-nitrophenyl phosphate (alkaline phosphatase substrate) solution (Sigma Chemical Co., St. Louis, MO, USA). Optical Density (OD) was monitored at 405 nm. Subjects were anti-NMDA receptor autoantibody positive, based on the cut-off value of 2 standard deviations above the mean OD of the healthy unrelated controls.

Anti-dsDNA testing

An indirect immunofluorescent assay (Inova Diagnostics, Inc., San Diego, USA) using the hemoflagellate *Crithidia luciliae* as a substrate was used to detect anti-dsDNA antibodies.

Statistics

The statistical analysis was performed using standard spreadsheet software and SPSS for Windows version 12, 2003 (SPSS Inc., Chicago, IL, USA). As OD values were not normally distributed in normality tests, Kruskal-Wallis test and Mann-Whitney test were applied to test for differences between independent groups. The presence of autoantibodies was compared between groups by chi-square test (or Fisher's exact test where appropriate). *P* value ≤ 0.05 was considered significant. All *P*-values are with a Mann-Whitney Test unless otherwise indicated.

Results

The group of SLE patients consisted mainly of women (94%) with a mean age of 40.7 (range 15-83) years. The mean age of the first-degree relatives (42.7; range 10-98 years) and healthy unrelated controls (41; range 12-68 years) was not different, but the percentage of females was only 57 and 38%, respectively. The overall SLEDAI score in the SLE population was 3.4 ± 4.0 (range 0-14), indicating little disease activity at the time of assessment (Table 1).

In 19 patients, neuropsychiatric syndromes were established. Different neuropsychiatric syndromes are listed in Table 2. Nineteen patients had a total of 30 neuropsychiatric syndromes.

Table 1. Patient characteristics and anti-NMDA values.*

	SLE patients (n = 51)	NP+ [a] (n = 19)	NP- [a] (n = 32)	First-degree relatives (n = 161)	Unrelated controls (n = 55)
Age, mean (SD) years	40.7 (15.7)	39.9 (14.6)	41.1 (16.6)	42.7 (16.7)	41 (13.2)
Female / male	48 / 3	17 / 2	31 / 1	91 / 70	21 / 34
SLEDAI, mean (SD)	3.4 (4.0)	4.7 (4.6)	2.5 (3.3)	-	-
Anti-dsDNA pos, n (%)	31 (61)	12 (63)	19 (59)	-	-
NMDA ab pos [b], n (%)	5 (10)	1 (5)	4 (13)	24 (15)	3 (6)
Value OD [c], mean (SD)	0.37 (0.16)	0.37 (0.1)	0.37 (0.19)	0.4 (0.18)	0.31 (0.12)

* NMDA, *N*-methyl-D-aspartate; SLEDAI, systemic lupus erythematosus disease activity index; anti-dsDNA, antibodies against double stranded DNA.

[a] NP+ / NP-, with / without neuropsychiatric symptoms.

[b] Positive reactivity = based on 2 x SD above the mean of unrelated controls.

[c] OD = Optical Density 405 nm.

Table 2. Neuropsychiatric manifestations in 19 SLE patients.*

Neuropsychiatric manifestations	n
Cognitive dysfunction	9
Cerebrovascular disease	8
Headache	5
Seizures	2
Myelopathy	2
Plexopathy	1
Mood disorder	1
Acute confusional state	1
Polyneuropathy	1

[*] Eleven patients with 1 syndrome, 5 patients with 2 syndromes, 3 patients with 3 syndromes.

Eleven patients had one syndrome, five patients had two syndromes and three patients had three syndromes.

Anti-NR2 antibody reactivity in serum is shown in Figure 1. There was a significant difference in mean antibody reactivity between SLE patients and healthy unrelated controls (mean OD value 0.37 ± 0.16 versus 0.31 ± 0.12, $P < 0.01$) and between first-degree relatives and healthy unrelated controls (mean OD value 0.40 ± 0.18 versus 0.31 ± 0.12, $P < 0.001$). No difference was found between lupus patients and their first-degree relatives ($P = 0.23$) or between lupus patients with and without neuropsychiatric symptoms (mean OD value 0.38 ± 0.10 versus 0.36 ± 0.18, $P = 0.16$). The mean OD value was low in all groups (0.31-0.40). In two SLE patients, the OD was > 0.8, they both had no neuropsychiatric manifestations.

On the basis of a cut-off value of two times the standard deviation above the mean OD value of the unrelated controls (leaving 6% of them autoantibody positive) 10% of SLE patients and 15% of relatives were anti-NMDA receptor antibody positive as is shown in Table 1. There was no significant difference in frequency of antibody-positive individuals between the three groups ($P = 0.15$ by chi-square test) and no difference was found between SLE patients with and without neuropsychiatric symptoms ($P = 0.64$ by Fisher's exact test).

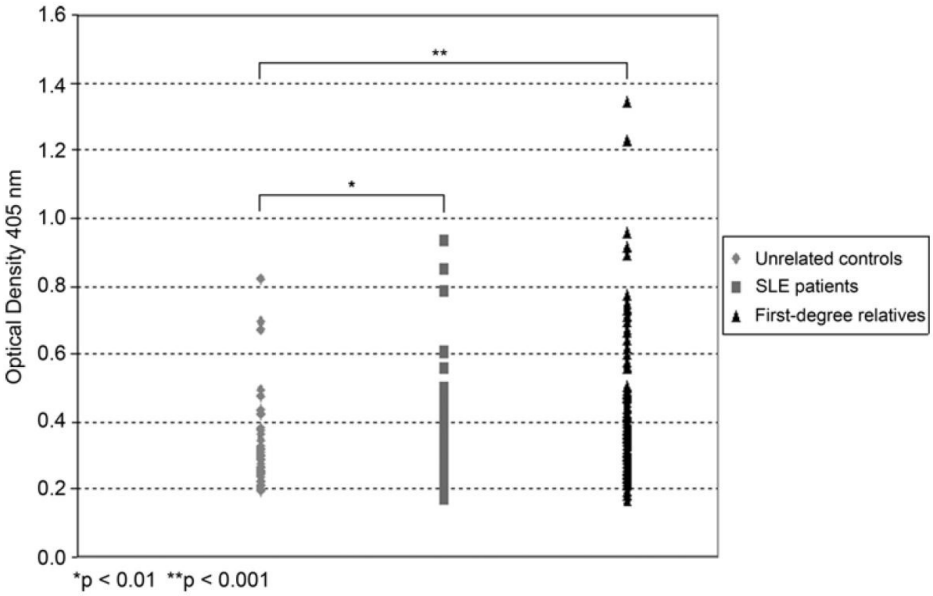

Figure 1. Anti-NR2 antibody ELISA performed in 51 SLE patients, 161 first-degree relatives and 55 unrelated controls.

Sixty-one percent of the SLE patients were positive for anti-dsDNA antibodies. No difference was found between patients with or without neuropsychiatric symptoms. All patients positive for anti-NMDA receptor antibodies were also positive for anti-dsDNA antibodies.

Discussion

Anti-NMDA receptor autoantibodies were detected in all groups. The frequency of anti-NMDA receptor autoantibody-positive individuals was not different between SLE patients, their first-degree relatives and unrelated controls. However, the levels of autoantibodies were significantly different between unrelated individuals and SLE patients as well as their first-degree relatives. No difference was found between patients with and without neuropsychiatric symptoms. The fact that higher levels of these antibodies are demonstrated in lupus patients as well as their first-degree relatives indicates a familial basis to mount an immune response to this peptide. This is in line with previous studies, which showed that the presence of autoantibodies, though not always directed against the same nuclear antigens, clusters within the families of SLE patients.[1,2]

Though the number of patients is probably too low to find associations between anti-NMDA receptor autoantibodies and neuropsychiatric symptoms in SLE patients, our results support the concept that the presence of anti-NMDA receptor autoantibodies in serum alone is not

associated with neuropsychiatric symptoms. This is in line with data from Husebye and colleagues who found a significantly higher percentage of SLE patients positive for anti-NMDA receptor autoantibodies (31%) compared to patients with other autoimmune diseases and healthy donors, but found no correlation between the presence of these antibodies and any lupus related clinical manifestation.[19] However, a recent study did show an association between elevated anti-NMDA receptor autoantibody levels in plasma samples and impairment of cerebral functions (depressed mood, decreased short-term memory and learning) in a group of 57 SLE patients, suggesting a direct effect of these antibodies.[20] Lapteva *et al*.[21] confirmed the association with these autoantibodies and depressive mood but not with cognitive dysfunction, but Harrison *et al.*[22] at the same time found no association with both syndromes nor with anxiety.

In this context, much interest is arising in the role of damage to the blood-brain barrier, which could be influenced by different factors such as hormones, cytokines and steroid therapy.[23] Accordingly, the integrity of the blood-brain barrier could be an important factor in the pathogenesis of neuropsychiatric manifestations in SLE as was demonstrated by Kowal *et al*.[15] In this mouse model, neuronal damage occurred only when a breach in the integrity of the blood-brain barrier was present. Furthermore, an association was found between IgG anti-NR2 glutamate receptor antibodies in CSF with neurologic syndromes of the central nervous system (CNS) in SLE patients, suggesting that it might be more useful to measure these autoantibodies in CSF than in serum samples for the diagnosis of NPSLE.[24]

The antigenic mimicry between epitopes on DNA and the NMDA receptor has been suggested by the correlation of anti-NMDA receptor reactivity with anti-dsDNA reactivity in 2001. However, other studies such as the study by Husebye *et al.* showed no correlation between these two antibodies.[19] The precise nature of this topic is a matter of future research. In this study, we did not test anti-dsDNA antibodies in first-degree relatives and controls. Though it is unknown whether the antigen represented by the linear epitope (containing the pentapeptide sequence DWEYS) of the NR2 subunit of the NMDA receptor is the physiologically relevant epitope for testing for the autoantibody-induced NPSLE, it is the only more or less established test and was therefore used in this study. In general, a standard curve for relative levels of antibodies is used to minimize assay to assay variability. Still, the OD values reported here could be used for the comparison of antibody levels between the three groups since the samples were randomly distributed over the 96-well plates, thereby minimizing technical reasons for the observed differences in OD. We included healthy controls on all plates thereby trying to minimize variation caused by possible different time periods that the substrate was converted by alkaline phosphatase. Hence both the strict adherence to 90 minutes before blocking the reaction and the inclusion of healthy controls on all plates minimizes possible artefacts by using ODs.

Overall OD values were low. Differences in the levels of autoantibodies may be influenced by gender, age and the number of freeze-thaw cycles of the plasma samples. Handling and storage of the plasma samples used to perform antibody assays is a potential source of assay variability. Brey *et al.*[25] evaluated the effect of repeated freeze-thaw cycles on anticardiolipin antibody

levels using ELISA and found a significant decline in the aliquot that had been through three cycles. In contrast, other investigators found no significant effect on measured antibody levels or the ability to measure these antibodies by ELISA after completing 10 freeze-thaw cycles.[26] As all our samples had been through a maximum of two freeze-thaw cycles before, a general decline in the antibody levels could have occurred, but is less likely.

A limitation of this study is that neuropsychiatric manifestations were classified retrospectively. We realize that the ACR nomenclature and case definitions for neuropsychiatric lupus syndromes is not validated to be used retrospectively, but this nomenclature is the most extensive attempt to classify clinical manifestations of NPSLE and as it was not adopted yet at the time these patients were diagnosed with NPSLE, we applied it retrospectively as complete as possible. This may lead to a bias as milder symptoms may not have been recorded and written down in the patient file, leading to a selection of more severe neuropsychiatric manifestations. This adds to the specificity, but leads to an underestimation of the incidence of milder neuropsychiatric symptoms. This is especially true for some of the clinical syndromes such as headache and cognitive dysfunction that have a low specificity. It is not clear whether a unique headache syndrome exists that is attributable to SLE. We classified a patient as having headache as an NPSLE syndrome only if the headache was repetitively referred to by the patient, explicitly written in the file, judged as a serious problem by the treating physician and after exclusion of other possible diseases and underlying causes. In patients with suspected cognitive dysfunction, neuropsychological testing was performed. No cognitive problems were reported by the first-degree relatives and the controls and neuropsychological testing was not performed in these subjects. In this way, we may also have underestimated the incidence of subclinical disease or subtle neuropsychiatric symptoms in these groups.

We conclude that in our study anti-NR2 autoantibody reactivity is higher in SLE patients and first-degree relatives, but no difference was found between subjects with or without neuropsychiatric symptoms. Therefore the tendency to mount this immune response seems a familial trait. Further studies whether these antibodies induce neuropsychiatric dysfunction in humans, as in mice, are needed.

Acknowledgement

The authors thank B. Diamond of the Department of Microbiology & Immunology and Medicine, Albert Einstein College of Medicine in New York, for analyzing the plasma samples.

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