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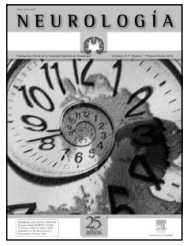

ORIGINAL ARTICLE

Clinical spectrum associated with aquaporin-4 antibodies (NMO-IgG)

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Clinical spectrum associated with aquaporin-4 antibodies (NMO-IgG)

Abstract
Introduction: The description of a highly sensitive and specific biomarker for neuromyelitis optica (NMO-IgG/aquaporin-4 antibody) extended the clinical spectrum of NMO to limited forms such as optic neuritis (ON) and longitudinally extensive myelitis (LEM).
Objective: To asses the sensitivity and specificity of our assay, and to describe the clinical characteristics of the patients who were tested for NMO-IgG.
Methods: NMO-IgG was analysed by immunohistochemistry and confirmed by assay on HEK cells transfected with aquaporin-4. The clinical information was obtained from forms filled in by the referring neurologists.
Results: A total of 580 samples from 518 patients were analysed from November 2005 to September 2008. Clinical information was available from 358 (68%) patients. All 33 (100%) positive cases were followed up. Twenty-eight of the 43 (65%) patients diagnosed with NMO by the revised criteria of 2006 were positive; the sensitivity was 62.5% when applying the same criteria, but discounting the criterion of NMO-IgG status, or 57% when applying the criteria of 1999. NMO-IgG was detected in 3 (13%) of the recurrent LEM and 2 (4%) of the recurrent ON. NMO-IgG was not detected in the remaining patients (96 with a final diagnosis of multiple sclerosis; 80 with myelitis; 28 with non-recurrent ON; and 33 other diagnosis).
Conclusions: No false positive cases were found in this large and non-selected study. NMO-IgG positive cases were mostly associated with NMO, and only in a low percentage with recurrent ON or LEM.

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PALABRAS CLAVE
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Neuritis óptica

Espectro clínico asociado a anticuerpos contra aucoporina 4 (IgG-NMO)

Resumen
Introducción: Los anticuerpos IgG-NMO se han demostrado sensibles y específicos para el diagnóstico de neuromielitis óptica (NMO) y han permitido ampliar el espectro clínico a formas limitadas como neuritis óptica (NO) o mielitis longitudinalmente extensas (MLE).
Objetivo: Evaluar la sensibilidad y la especificidad de nuestra técnica y describir las características de los pacientes para los que se solicita dicha determinación.
Métodos: Los anticuerpos IgG-NMO se analizaron mediante inmunohistoquímica y se confirmaron sobre células HEK transfectadas con acuaporina 4. La información clínica se obtuvo mediante un cuestionario rellenado por el neurólogo remitente de la muestra.
Resultados: Desde noviembre de 2005 a septiembre de 2008 se analizaron 580 muestras de 518 pacientes. Se obtuvo información de 358 (68%) pacientes. El seguimiento en los 33 casos positivos fue del 100%. De los 43 pacientes diagnosticados de NMO por los criterios de 2006, 28 (65%) eran positivos; la sensibilidad fue del 62,5% si se aplicaban estos criterios eliminando el resultado de IgG-NMO y del 57% aplicando los criterios de 1999, que tampoco incluyen los IgG-NMO. Se detectaron IgG-NMO en 3 (13%) de las MLE recurrentes y 2 (4%) de las NO recurrentes. No se detectaron IgG-NMO en el resto de los pacientes evaluados (96 finalmente diagnosticados de esclerosis múltiple; 80 mielitis; 28 NO no recurrentes; 33 con otros diagnósticos).
Conclusiones: En este estudio no seleccionado y tan amplio, no se han detectado falsos positivos. Los casos positivos se asocian mayoritariamente con NMO y sólo en un pequeño porcentaje con NO o MLE recurrente.

Introduction

Neuromyelitis optica (NMO) is an inflammatory demyelinating disease, usually severe, which predominantly affects the optic nerve and spinal medulla[1]. The discovery of a specific serological marker[2,3], NMO-IgG or aquaporin-4 antibodies, has enabled: *a*) the expansion of the clinical spectrum to include partial or limited forms of the disease such as recurrent optic neuritis (ON) or longitudinally extensive myelitis (LEM)[1,2,4-6]; *b*) the proposal of new diagnostic criteria that recognise clinical manifestations or through magnetic resonance imaging (MRI) outside the optic nerve or the medulla[7,8], and *c*) the confirmation that it is disease with a different etiopathogenesis from that of multiple sclerosis[9-11].

The prevalence of the disease is poorly understood, although it is considered a rare disease in Caucasian populations, with a rate below 1% of cases of demyelinating diseases[1]. Depending on the technique used in the analysis of NMO-IgG and the diagnostic criteria applied, there have been descriptions of up to 10-46% of patients with NMO being seronegative and that false positives can reach up to 10%[2,13]. The availability of a technique may cause an increase in the number of patients in whom the disease is suspected, which could lead to more cases being diagnosed. However, it could also generate an increase in the number of false positives inherent to the diagnostic technique.

In our laboratory, the determination of NMO-IgG began in November 2005. The aim of this study was to evaluate the sensitivity and specificity at our laboratory and to describe the characteristics of patients for whom this determination was sought.

Patients and methods

We reviewed the database of samples sent to the Neuroimmunology Laboratory of the Barcelona Hospital Clinic for the determination of NMO-IgG between November 2005 and June 2008. Clinical data were collected through a survey sent by the requesting neurologists through an established template or by telephone interview. This survey described, in addition to demographic variables, the items included in the diagnostic criteria for NMO from 1999[14], the number and the type of episodes, concomitant autoimmune diseases, treatments undergone and the score on the Kurtzke expanded disability status scale (EDSS)[15] at the last follow-up and in diagnosis.

The definitive diagnosis of NMO was established by applying the updated 2006 criteria[7]; patients with acute transverse myelitis and medullar MRI with lesion $\geq$ 3 vertebral bodies were classified as LEM; the diagnosis of multiple sclerosis (MS) was established following the McDonald criteria[16]; and that of ON and other diagnoses was based on the final diagnosis of the referring physicians.

NMO-IgG was determined by immunohistochemistry using frozen sections of rat hippocampus and a technique of avidin-biotin-peroxidase (1:500 serum dilution) as was described previously[8,17]. All positive cases were confirmed by immunohistochemistry on HEK cells transfected with

aquaporin-4 (AQP4). Briefly, HEK293T cells grown to a 70% confluence were transfected with the AQP4 gene, cloned into the pEGFP-C1 vector (BD Biosciences Clontech). Lipofectamine 2000 (Invitrogen) was used for this purpose, following the manufacturer's instructions; specifically, 2 µl of Lipofectamine 2000 were combined with 0.8 µg of DNA in each well in a P24 plate. After 40 h of transfection, cells were fixed with 4% paraformaldehyde in PBS and incubated sequentially with patient serum and an appropriate secondary antibody (Fig. 1).

Statistical analysis

Qualitative variables were compared through the chi-square or the Fisher tests; for quantitative variables, Student's t-test or Mann-Whitney U test were used, depending on the conditions of application. A value of $p < 0.05$ (bilateral significance) was considered statistically significant. The analysis was performed using the statistics package SPSS version 16.0.

Results

During the period indicated, 580 samples from 518 patients were analysed. Information was obtained from 358 (68%) patients. The monitoring was 100% in the 33 positive cases. All positive cases by conventional immunohistochemical were confirmed through study with transfected HEK cells.

Forty-three patients were diagnosed with NMO, 103 with myelitis (59 LEM), 77 with ON, 96 with multiple sclerosis (MS) and 33 had other diagnoses. NMO-IgG was detected in 28 (65%) of the NMO cases, in 3 (13%) of the recurrent LEM cases and in 2 (4%) of the non-recurring. The NMO-IgG were negative in the remaining patients analysed by conventional immunohistochemistry.

Table 1 shows the characteristics of patients with NMO based on NMO-IgG result. The only significant difference was the predominance of women in the seropositive group. If the revised criteria of 2006[7] were implemented but eliminating the NMO-IgG criterion, the sensitivity of the technique was 62.5% (95% confidence interval [CI], 47% 78%). If the earlier 1999 criteria[14], which also excluded

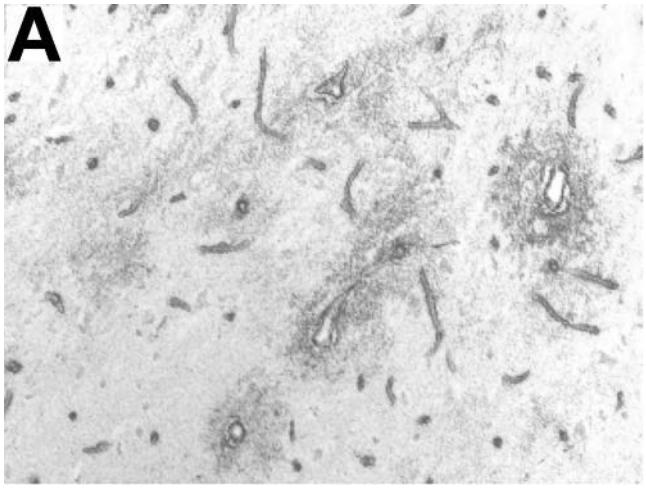

Figure 1 A: conventional immunohistochemistry; the pattern of immunoreactivity characteristic of NMO-IgG that mark the microvessels of the white matter of the rat hippocampus can be observed. B: HEK cells transfected with the gene for aquaporin-4, cloned into the vector pEGFP-C1 (green fluorescence) (B1), and serum from a patient with positive NMO-IgG by conventional immunohistochemistry (red fluorescent secondary antibody) (B2).

NMO-IgG, were applied, the sensitivity was 57% (95% CI, 43%-71%).

As expected, the majority of patients for whom the determination of NMO-IgG was requested were finally diagnosed with MS. As a group, patients with MS were significantly different from those with NMO in all variables analysed. Age at onset was lower, severe clinical manifestations (ON or severe weakness) were less frequent, extensive lesions were more frequent in the spinal MRI (≥ 3 vertebral bodies) and the average of relapses was lower, while oligoclonal bands were present in the cerebrospinal fluid (CSF) more frequently. In contrast, no significant differences were found in the in EDSS (Table 2).

Of the 103 myelitis cases, 59 (57%) were LEM, of which 23 (39%) had presented some recurrence. The characteristics of the patients with myelitis, in terms of whether recurrences were presented or not, are listed in Table 3. As can be seen, there were no significant differences between the two groups. The rest of the myelitis cases (44) were from patients with partial myelitis, of which 29 (66%) had suffered recurrences (data not shown).

Of the 77 ON cases, 49 (64%) were recurrent. The characteristics of the patients are presented in Table 4. Compared with patients with single-phase ON, patients with recurrent ON were significantly younger at disease onset, showed a higher frequency of bilateral ON and evidenced a tendency towards a higher frequency of cerebral MRI with a result that was negative or not diagnostic of MS.

The low number of patients with positive NMO-IgG and LEM (3) or ON (2) prevented a statistical comparison with seronegative patients.

Other diagnoses included patients with acute disseminated encephalomyelitis, Bickerstaff's brainstem encephalitis, necrohaemorrhagic myelitis, spinal tumour, syringomyelia, progressive myelopathy, meningeal carcinomatosis, toxic optic neuropathy, diabetic neuropathy/retinopathy, progressive bilateral optic neuropathy, ischemic ON and other unspecified disorders.

Three seropositive patients were analysed after immediate plasma exchange. In 2 cases the analysis was negative. Only one of these was analysed again in connection with a new outbreak, with a positive result. Nine seropositive patients were analysed on more than one occasion (median, 2; range, 2-6), with no changes being found in the result. The number of seronegative patients who were analysed more than once was very low, and in most cases no clinical information was available. It was also rare to have paired samples of serum and CSF, and only in one case was the serum positive while the CSF was negative.

Discussion

The most comprehensive non-selected study described in the literature, our study has analysed the presence of NMO-IgG antibodies in patients with suspected NMO or related clinical syndromes. Our most relevant result is the lack of false positives with the technique used. Knowing this data is important, because a positive result can therefore give us confidence that it is a NMO or disease in its clinical spectrum, and this fact helps us make a treatment decision that is often not without risk.

The study confirms that MS is the disease that most often presents a differential diagnosis with NMO[1,2,8]. Both diseases as a group are significantly different in demographic, clinical, radiological and immunological parameters;

Table 1 Demographic and clinical characteristics of patients with neuromyelitis optica with respect to NMO-IgG

	Positive NMO-IgG (n = 28)	Negative NMO-IgG (n = 15)	p
Age at onset (years), median (range)	34 (14-62)	38 (21-68)	0.64
Age of evaluation (years), median (range)	41 (23-62)	42 (21-78)	0.81
Gender (female/male)	25/3 = 8,3	19/8 = 2,4	0.046
Initial syndrome, n (%)			
ON	13 (48)	8 (53)	0.57
Myelitis	9 (33)	6 (40)	
Myelitis + ON	5 (18)	1 (7)	
Negative brain MRI, n (%)	27 (100)	15 (100)	—
Spinal MRI ≥ 3 vertebral bodies, n (%)	25 (93)	15 (100)	0.53
Severe ON (< 20/200), n (%)	20 (77)	7 (54)	0.16
Bilateral ON, n (%)	21 (75)	6 (43)	0.08
Severe weakness, n (%)	24 (89)	11 (79)	0.39
Total outbreaks, median (range)	5 (2-15)	3 (2-10)	0.07
EDSS, median (range)	4 (1-8,5)	3,5 (1-8)	0.59
OCB present, n (%)	2 (9)	3 (23)	0.34
ANA and/or ENA autoantibodies present, n (%)	4 (16)	3 (21)	0.54

ANA: antinuclear antibodies; EDSS: Kurtzke expanded disability status scale[15]; ENA: extractable nuclear antigen; MRI: negative brain magnetic resonance image (normal or not complying with the Paty criteria)[14]; OCB: IgG oligoclonal bands; ON: optic neuritis

however, raising this differential diagnosis is logical because approximately half of all MS patients in this series showed no symptoms outside the optic nerve and the marrow, 40% had a normal or non-diagnostic brain MRI and up to 20% presented an extensive lesion in the spinal MRI. Taking into account the different prognosis in both diseases and the fact that standard therapy for MS is immunomodulatory whereas for NMO it is immunosuppressant[1], it is not surprising that NMO-IgG identification is requested for patients who finally end up being diagnosed with MS.

The frequency of NMO-IgG detection (57-65%) in our study, based on the diagnostic criterion of NMO, is within the range described in other studies (54-73%) that analysed smaller-sized samples (median, 25; interval, 11-45)[12,13]. In a previous study, we demonstrated that our immunohistochemical technique was as sensitive as the original immunofluorescence

Table 2 Demographic and clinical characteristics of patients diagnosed with neuromyelitis optica and multiple sclerosis

	NMO (n = 43)	EMS (n = 96)	p
Age at onset (years), median (range)	34 (14-68)	29 (8-58)	0.003
Edad de evaluación (años), mediana (intervalo)	42 (21-78)	35 (19-72)	0.012
Gender (female/ male)	35/ 8 = 7	62/ 34 = 1,8	0.046
Initial syndrome, n (%)			
ON	21 (50)	28 (29)	0.01
Myelitis	15 (35)	49 (51)	
Myelitis + ON	6 (14)	4 (4)	
Optic neuritis, n (%)	43 (100)	68 (71)	0.0001
Myelitis, n (%)	43 (100)	87 (91)	0.06
No other condition, n (%)	39 (93)	45 (55)	0.0001
Negative brain MRI, n (%)	42 (100)	36 (41,4)	0.0001
Spinal MRI ≥ 3 vertebral bodies, n (%)	40 (95)	19 (20)	0.0001
Severe ON (< 20/ 200), n (%)	29 (69)	17 (21,5)	0.0001
Bilateral optic neuritis, n (%)	32 (64)	21 (24)	0.0001
Debilidad severa	35 (85)	34 (58)	0.004
Total outbreaks (neuritis and/ or myelitis), median (range)	4 (2-15)	2 (0-8)	0.0001
EDSS, median (range)	4 (1-8,5)	2,5 (0-8)	0.006
OCB present, n (%)	5 (14)	39 (60)	0.0001
Autoanticuerpos ANA y/ o ENA presentes, n (%)	6 (15)	6 (12,5)	0.3

ANA: anticuerpos antinucleares; BOC: bandas oligoclonales de IgG; EDSS: escala de discapacidad ampliada de Kurtzke[15]; ENA: antígeno extraíble del núcleo; NO: neuritis óptica; RM: resonancia magnética cerebral negativa (normal o no cumple criterios de Paty)[14].

Table 3 Características demográficas y clínicas de los pacientes diagnosticados de mielitis longitudinalmente extensa

	LEM (n = 36)	RLEM (n = 23)	p
Age at onset (years), median (range)	36 (25-72)	47 (14-70)	0.42
Age of evaluation (years), median (range)	36 (25-77)	48 (14-70)	0.77
Gender (female/ male)	23/ 13 = 1,8	17 = 2,8	0.57
No other condition, n (%)	28 (96,6)	16 (80)	0.14
Negative brain MRI, n (%)	21 (95,5)	16 (80)	0.17
Spinal MRI ≥ 3 vertebral bodies, n (%)	36 (100)	23 (100)	—
Severe weakness, n (%)	21 (81)	16 (89)	0.68
Total outbreaks, median (range)	—	2 (1-5)	—
EDSS, median (range)	5 (0-8,5)	3,5 (1-8)	0.98
OCB present, n (%)	1 (6)	6 (33)	0.09
ANA and/ or ENA autoantibodies present, n (%)	2 (13)	1 (7)	1

ANA: antinuclear antibodies; OCB: IgG oligoclonal bands; EDSS: Kurtzke expanded disability status scale[15]; ENA: extractable nuclear antigen; MRI: negative brain magnetic resonance image (normal or not complying with the Paty criteria)[14].

Table 4 Demographic and clinical characteristics of patients diagnosed with optic neuritis

	NO monofásica (n = 28)	RON (n = 49)	p
Age at onset (years), median (range)	46 (16-59)	32 (10-45)	0,011
Age of evaluation (years), median (range)	46,5 (18-64)	36,5 (17-58)	0,012
Age of evaluation (years), median (range)	20/ 8 = 2,5	28/ 21 = 1,3	0,23
Gender (female/ male)	19 (86)	38 (81)	0,7
No other condition, n (%)	16 (80)	43 (96)	0,067
Severe ON (< 20/ 200), n (%)	14 (82)	28 (70)	0,51
Bilateral ON, n (%)	12 (48)	34 (77)	0,018
Total outbreaks, median (range)	—	2 (1-6)	—
EDSS, median (range)	3 (0-4)	2 (0-4)	0,93
OCB present, n (%)	1 (7)	2 (6)	0,87
ANA and/ or ENA autoantibodies present, n (%)	1 (7)	3 (10)	0,72

ANA: antinuclear antibodies; EDSS: Kurtzke expanded disability status scale[15]; ENA: extractable nuclear antigen; MRI: negative brain magnetic resonance image (normal or not complying with the Paty criteria)[14]; OCB: IgG oligoclonal bands; ON: optic neuritis; RON: recurrent optic neuritis.

technique[8]. In the current study, samples of patients with seronegative NMO were reanalysed at a lower dilution (1:100) with no new positive cases being detected. Recent studies have shown that the immunohistochemical technique on HEK cells transfected with AQP4 is more sensitive than the conventional technique[18,19]. In one study the detection rate in patients who met the reviewed criteria for NMO from 2006 increased from 58 to 80%[18]. In our laboratory, we have begun to standardise the technique, and we have confirmed the result of all samples positive by conventional immunohistochemistry using it. Our immediate objective will be to verify whether we can increase the sensitivity of this technique without affecting the specificity.

The characteristics of patients with NMO in this series are similar to those in other series published[1,7,13]. NMO is between 7 and 9 times more prevalent in women than in men and the average age at onset is around the early thirties, an average of about 5 years higher than for MS. Brain MRI at the time of onset is normal or not diagnostic of MS, and spinal MRI shows lesions of more than 3 vertebral bodies in more than 95% of cases, while the presence of oligoclonal IgG bands in CSF is rare (14-20%). The only notable fact in an American series is our low rate of non-organ-specific autoantibodies, mainly anti-nuclear antibodies (ANA) and/ or extractable nuclear antigens (ENA) (15 vs. 50%)[7]. It is noteworthy that, as in the French series[13] and in our previous study, which included Italian patients[8], we have seen no significant differences between patients based on the presence or absence of NMO-IgG.

The current study confirms that NMO-IgG may also be present in patients with isolated LEM and ON but, as in other publications, associated only with recurrent forms. However, the detection rate in our study (13 and 4%, respectively) was low. In LEM series in which the number of patients evaluated is lower (median, 13; range, 6-29) the number of positives ranges between 38 and 100%[4,13,18,20]. Nevertheless, in these studies it is striking that the number of patients with NMO included is at least twice that of LEM, in contrast to this study: this indicates a possible selection of the test sample. In any case, the absence of clinical data in those studies precludes knowing whether our patients are different or not. In the case of ON, two studies with 24 and 34 patients found rates of positives of 25 and 20% respectively[5,6]. In both of those, it stands out that seropositive patients present more recurrences (average, 4) and a higher frequency of severe episodes (100%) than the patients in this series (2 and 70%, respectively).

Finally, we recognise the limitations of this study, which by its nature may have influenced the interpretation of results. For example, it was only possible to monitor 68% of patients, which may have influenced the sensitivity of our technique for NMO diagnosis. However, this does not affect specificity, because monitoring was obtained for 100% of positive cases. The study should be considered as transversal, given that monitoring of evolution (which might have changed the initial diagnosis) was carried out in few cases and the questionnaire was received after the completion of the analysis in many cases (which may have influenced the final diagnosis). Nor has there been a centralised assessment of tests such as MRI, in which the number and characteristics of injuries are of great diagnostic value for the entities under consideration, or that helps to determine whether the technical quality of the MRI used has been the most adequate in defining the outcome reflected in the survey.

In conclusion, no false positives have been detected in this broad study with non-selected patients with suspicion of NMO or related diseases. Further collaborative studies designed to answer the questions that this study could not answer are needed. For example, whether the test result is influenced by the patient's clinical condition (at the onset or stable phase)[21,22] or if a parallel CSF study can provide additional information[23].

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Conflict of interests

The authors declare no conflict of interests.

Presentations

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