Fine specificity of antibodies against AQP4: Epitope mapping reveals intracellular epitopes

E.I. Kampylafka a, J.G. Routsias a, H. Alexopoulos a, M.C. Dalakas a,b, H.M. Moutsopoulos a, A.G. Tzioufas a,*

a Department of Pathophysiology, Medical School, National and Kapodistrian University of Athens, 75 Mikras Asias str., 11527 Athens, Greece
b Department of Neurology, Thomas Jefferson University, Philadelphia, PA, USA

1. Introduction

Neuromyelitis optica (NMO or Devic’s Disease) is a rare autoimmune inflammatory demyelinating syndrome of the Central Nervous System (CNS) that preferentially targets the optic nerves and spinal cord [1]. Its course is usually relapsing, but without marked remission between relapses, leading to rapid accumulation of irreversible deficits [2]. The studies by Lennon et al. [3] have provided unequivocal evidence that a specific autoantibody (NMO-IgG) against aquaporin-4 (AQP4) is a marker for the disease and differentiates neuromyelitis optica from multiple sclerosis [1]. Further studies have shown that anti-AQP4 antibodies are not only detected in neuromyelitis optica, but also in patients with the NMO spectrum of diseases [4] that includes limited forms of NMO [5], optic-spinal MS [6], and demyelinating conditions accompanying systemic autoimmune diseases, such as Systemic Lupus Erythematosus and Sjögren’s Syndrome [5].

Aquaporin-4 belongs to a family of water channels (AQP0-12) involved in water transport in different tissues [7]. It consists of 3 extracellular loops, 2 intracellular loops and the N- and C-terminal regions of the protein, that connect 6 alpha helices spanning the membrane [8]. It exists as heterotetramers composed by the M1 (M.W. 34 kDa) and M23 (M.W. 31 kDa) splice variants of aquaporin-4, which differ in their first 22 amino acids [9]. AQP4 is the most abundant water channel in the CNS and is highly expressed on the astrocytic endfeet at the blood–brain barrier (BBB), regulating water transport between blood, brain and cerebrospinal fluid [10].

Autoantibodies to AQP4 appear to possess a pathogenetic role, as shown by previous studies, since they induce surface redistribution and endocytosis of AQP4, complement activation, water homeostasis and blood–brain barrier disruption, leading eventually to the recruitment of inflammatory cells [11].
Furthermore, NMO-like disease was successfully induced after passive transfer in animal models, following BBB breakdown [12,13], or by direct intra-cerebral IgG injection [14]. Despite the fact that anti-AQP4 antibodies have an established role in the pathogenesis of neuromyelitis optica, their specificity regarding antigenic epitopes has not yet been determined. Some studies support the existence of conformation-dependent epitopes [15,16], although others suggest the recognition of linear epitopes in the denatured molecule [17]. The aim of the present study was to identify the B-cell linear epitopes of the AQP4 protein and to investigate antigenic similarities with other molecules.

2. Materials and methods

2.1. Patients and sera

We used sera from 21 patients positive for NMO IgG/anti-AQP4 antibodies, as detected by indirect immunofluorescence on mouse brain tissue [3] (21/21 positive) and by a cell based assay using M23-transfected (M23-eGFP clones were a kind gift from Dr. P. Waters and Prof. A. Vincent, University of Oxford) HEK293 cells [18] (16/21 positive). All patients belonged to the neuromyelitis optica spectrum of diseases [4] and presented active CNS involvement during blood sampling, as attested by the clinical picture and MR Imaging. Sera from 28 healthy subjects were used as normal controls. The disease control group comprised of 23 patients with Systemic Lupus Erythematosus (SLE) and 23 patients with primary Sjögren’s Syndrome (pSS), without neurological involvement. All SLE patients fulfilled the 1997 American College of Rheumatology revised criteria for the classification of Systemic Lupus Erythematosus [19], while the pSS patients fulfilled the American-European Consensus Group revised criteria [20].

2.2. Peptide synthesis

Eleven peptides, in the form of peptide dendrimers (multiple antigenic peptides, MAP) were synthesized by Bio-synthesis Inc, U.S.A. The peptides covered all the intracellular and extracellular domains of both AQP4 isoforms (M1: UniProtKB accession number P55087-1, M23: UniProtKB accession number P55087-2), and were either single peptides or overlapping by 8 amino acids. The first 2 peptides (AQPpep1 and AQPpep2) covered the N-terminus intracellular domain of the protein (Fig. 1). AQPpep1 was designed to include the first 22 amino acids that are included only in the M1 isoform of the protein in a non-overlapping fashion with AQPpep2 that starts exactly at the N-terminus of M23 isoform. Peptides AQPpep3–AQPpep7 corresponded to the three extracellular and two intracellular loops of the protein and, finally, the C-terminus intracellular domain of the protein was covered by four 24-mer peptides, overlapping by 8 amino acids (Peptides AQPpep8,
AQPepp9, AQPepp10 and AQPepp11). Two additional 15-mer peptides corresponding to the [aa257–271] of the AQP4 protein and the [aa219–233] of the TAX1BP1 (Tax1-HTLV-1 binding protein, UniProtKB accession number Q86VP1) were also synthesized (Table 1).

2.3. ELISA assays

All patients and controls were evaluated for the presence of autoantibodies against the 11 peptides by ELISA assays. 96-well microtitre plates were coated with 100 μl of peptide solution (2.5 μg/ml in sodium carbonate—sodium bicarbonate buffer, PH 9.8) and kept at 4 °C overnight. Afterwards, the remaining binding sites were blocked with 2% bovine serum albumin (BSA) in Phosphate Buffered Saline (PBS) at room temperature for 1 h. After 3 washes with PBS, sera were added in 1/150 in 2% BSA/PBS dilution. After a 2-h incubation and 3 washes with PBS, alkaline phosphate conjugated anti-human IgG, diluted 1/1200 in 2% BSA/PBS, was added for 1 h at room temperature. After the addition of alkaline phosphate substrate, color development was quantified at 405 nm. The cutoff values for each peptide assay were determined using the mean Optical Density (OD) plus 3 standard deviations of the sera from the 28 healthy controls.

Homologous inhibition experiments were performed, in order to evaluate the specificity of the potential epitopes. Sera from 3 NMO positive patients, as well as a pSS and an SLE patient, that exhibited high reactivity against the peptides, were selected. Serum was pretreated in 1/300 dilution with increasing concentrations (0 μg/ml, 10 μg/ml and 40 μg/ml) of peptides AQPepp1, AQPepp4, AQPepp8 (being the 3 most reactive peptides) and control peptide (Ctrl-Pep), and incubated overnight at 4 °C. Peptide AQPepp9 was chosen as a control, since it exhibited low reactivity during the first series of experiments. Subsequently, all sera dilutions were tested by ELISA for reactivity against the peptides. Inhibition percentages were calculated as ([OD 0 μg/ml] – [OD 40 μg/ml])/[OD 0 μg/ml] × 100% for each serum. All the steps of the assay were as described in Section (2.3), apart from the incubation of the sera dilutions that lasted 1 h instead of 2.

2.4. Homology search

The antigenic peptide sequences were compared against the UniProtKB database (version 2010_10). The similarity search was performed using the NCBI BLASTP (ver. 2.2.17) algorithm and scored with PAM30 matrices. A 73% sequence similarity was observed between AQPepp8 (amino acids 257–271) EFKKRFKEAFSKAAQ and the aa219–233 domain of the human protein TAX1BP1 (EFKKRFSDATSKAHQ) (Table 1). None of the other peptides showed any similarity with non-related to aquaporin human, bacterial or viral proteins.

2.5. ELISA assays to examine the potential cross-reactivity between AQP4 and TAX1BP1 15-mer peptides and heterologous inhibition assays

19 NMO positive patients and 19 normal controls were tested for the binding of autoantibodies against the two 15-mer peptides (AQPepp8’ [aa257–271] of the AQP4 protein and TAX1BP1pep [aa219–233] of the TAX1BP1) on plates coated with 5 μg/ml of each peptide in sodium carbonate—sodium bicarbonate buffer, PH 10.5. ELISA was performed as described above, Section (2.3). In order to investigate whether the two 15-mer peptides could cross-inhibit one another, a second series of inhibition assays was performed in liquid phase. 3 selected NMO positive sera that exhibited high reactivity against the 2 peptides were pretreated in dilution 1/400 overnight at 4 °C with increasing concentrations of each peptide separately, as well as control AQPepp9 (0 μg/ml, 10 μg/ml, 40 μg/ml). Subsequently, all sera were tested by ELISA as in Section (2.3), with the sole exception of incubation of the sera dilutions that lasted 50min.

2.6. Statistical analysis

All comparisons between groups of sera were performed using Fisher’s exact test. Spearman’s rank correlation coefficient (r_s) was used to assess statistical dependence between reactivity rates of NMO positive sera against the two 15-mer peptides.

3. Results

3.1. Identification of the antigenic sites and inhibition assays

Peptides AQPepp1, 2, 4, 5 and 8 were identified as being the most reactive ones in an initial epitope mapping experiment in which all 11 peptides were tested. The ELISA assay was performed using sera from ten NMO positive patients and eight healthy controls. Sera from healthy donors showed no reactivity against any of the peptides. Subsequently, all sera from NMO positive patients (N = 21) and healthy controls (N = 28) were tested against the peptides, which exhibited the highest reactivity in the initial experiment (AQPepp1, 2, 4, 5 and 8). Peptides AQPepp1, AQPepp4 and AQPepp8 contained the epitopes that were mostly recognized by the NMO positive sera, reaching reactivity levels of 42.9%, 33.3% and 23.8% of patients, respectively (Fig. 2A). 5 sera (23.8%) were positive for antibodies against all 3 peptides, 2 (9.5%) were positive for antibodies against peptides 1 and 4, and 2 more sera were positive for antibodies only against peptide 1.

The 3 most reactive peptides (AQPepp1, AQPepp4 and AQPepp8), are part of the intracellular regions of the molecule (Fig. 2B). These peptides were further tested against 23 SLE and 23 pSS disease controls, in order to assess the specificity of their recognition. SLE patients were found positive for antibodies against peptides AQPepp1, AQPepp4 and AQPepp8 in 13%, 43% and 8.7%, respectively, while 8.7%, 43% and 7.8% of pSS patients also recognized peptides AQPepp1, AQPepp4 and AQPepp8.

The proportion of NMO positive sera binding against peptides AQPepp1, AQPepp4 and AQPepp8 was statistically significantly higher than the proportion in healthy control individuals (P < 0.05). Antibodies against AQPepp1 and AQPepp4 also binded in a statistically significant manner in comparison to sera from disease controls (Fig. 3). The specificity of binding was assessed by homologous inhibition assays, which produced high inhibition rates in NMO positive sera that reached 84.3%, 71.1% and 84% for peptides AQPepp1, AQPepp4 and AQPepp8, respectively. Average inhibition rates, at 40 μg/ml of inhibitor, were 71.2% for AQPepp1, 67.9% for AQPepp4 and 66.9% for AQPepp8. On the other hand, inhibition rates with the control peptide (AQPepp9) were significantly lower, ranging from 11% to 45.3% (Fig. 4). Inhibition assays using disease control sera showed minimal inhibition rates, ranging from 3% to 15.1%, that were similar to the ones produced when the control peptide was used (3.1–9.3%).

Table 1

<table>
<thead>
<tr>
<th>Origin</th>
<th>Sequence</th>
<th>Amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQPepp8 (AQP4 protein)</td>
<td>EFKKRFKEAFSKAAQ</td>
<td>[aa5–20]</td>
</tr>
<tr>
<td>EFKKRF - A SSKA Q</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAX1BP1 protein</td>
<td>EFKKRFSDATSKAHQ</td>
<td>[aa219–233]</td>
</tr>
</tbody>
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*“-* symbol indicates conservative changes in amino acid sequence.
3.2. Sequence similarities of the immunodominant epitopes

The identification of the 3 most reactive peptides, which are therefore potential linear antigenic epitopes of the AQP4 molecule, enabled the search of protein databases to identify similar sequences with other unrelated proteins. A 73% sequence similarity was observed between AQPpep8 (amino acids 257–271) EFKRRFKEAFSKAAQ and the [aa219–233] domain of the human protein TAX1BP1 (EFKKRFSDATSKAHQ) (Table 1), which is involved in the replication of the HTLV-1 virus, the etiological agent of HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP).

3.3. Reactivity of the patients’ sera against the AQP4 and the TAX1BP1 15-amino acid peptides and inhibition experiments

These observations prompted us to design two 15-mer peptides containing the previously mentioned sequences (Table 1), and test them against the NMO positive sera and sera from healthy controls. Reactivity against the AQPpep8 and the TAX1BP1pep peptide was detected in 26.3% (N = 5) and 31.6% (N = 6) of NMO positive sera, respectively. All NMO-IgG-positive sera that were positive for antibodies against the AQPpep8, were also positive for antibodies against the TAX1BP1pep, while one patient was positive for antibodies against the TAX1BP1pep (Fig. 5A). Spearman’s rank correlation coefficient was high (r_s = 0.081, P < 0.0001). None of the normal controls exhibited any reactivity against any of the 2 peptides.

The potential of anti-AQPpep8 antibodies to cross-recognize the TAX1BP1pep was explored by homologous and cross-inhibition experiments. Pretreatment of the NMO positive sera with AQPpep8 and TAX1BP1pep resulted in a 60.1% and 36% inhibition of antibody binding to the AQPpep8 peptide (homologous and heterologous
inhibition, respectively). On the other hand, binding to TAX1BP1-pep, was reduced by 70% after pretreatment with the TAX1BP1-pep, and by 58.5% after pretreatment with AQPpep8. By contrast, control peptide (AQPpep9) did not inhibit AQPpep8 and TAX1BP1pep binding (Fig. 5B).

4. Discussion

Since the discovery of anti-AQP4 antibodies as markers and pathogenetic factors in NMO, a great variety of methods have been developed for measuring anti-AQP4 antibodies in NMO patients with differences in sensitivity and specificity. This diversity denotes differences to the nature of the antigen used for detection.

The original and most commonly used detection method is the indirect immunofluorescence assay (IIF) against mouse brain sections in which the antigen is presumably in its native form [3]. Certain studies report that the autoantibodies target epitopes on the extracellular regions of the AQP4 molecule in a conformation-dependent manner, since they recognize AQP4 only in the form of orthogonal arrays of particles (OAPs) [15]. In other studies, using ELISA and western blots that utilize denatured proteins as substrates, it is linear structures that are mainly detected [17]. Therefore, it is highly likely that both conformational and linear epitopes are targets of a polyclonal response against the aquaporin-4 autoantigen, as described for the majority of the autoantibodies [21].

In order to identify the fine specificity of autoantibodies targeting aquaporin-4, we used overlapping synthetic peptides covering all the extracellular and intracellular domains of the molecule. Such peptides can effectively mimic linear and partially conformational epitopes and have been used in the past to map the specificity of autoantibodies found in different autoimmune diseases, including anti-Sm [22], anti-U1 snRNP [23], anti-Ro/SSA [24], anti-La/SSB [25], antibodies against citrullinated a-enolase [26], and against anti-glutamic acid decarboxylase (GAD) 65 [27]. 11 peptides were synthesized, covering all the intracellular and extracellular potential binding sites of the anti-AQP4 antibodies. Screening the NMO-IgG-positive sera for antibodies against these peptides, identified 3 dominant epitopes on the N-terminus, the first intracellular loop and the C-terminus part of the protein. Disease control sera exhibited only minimal reactivity against the peptides. This reactivity can be attributed to humoral polyreactivity commonly observed in the sera of patients with autoimmune diseases. As 42.9% of the NMO positive sera recognized at least one of the 3 peptides, a large body of specific autoantibodies is probably
directed against linear epitopes of the AQP4 protein. The inhibition assays, verified the specificity of the recognition.

Interestingly, the most reactive of the 3 epitopes corresponded to the first 22 amino acids of the AQP4, which exist only in the M1 and not in the M23 isoform of the protein, suggesting that the M1 isoform exhibits definite antigenic sites. This finding agrees with Marnetto et al. [17] who showed, using western blot, that the major target of anti-aquaporin-4 antibodies in NMO is the linear AQP4-M1 isoform. Our observation also supports the recent finding of two different missense allelic mutations at Arg19 (R19I and R19T), which enhance susceptibility to NMO and are located within the first 22 residues of the N-terminus of the protein, unique to the AQP4-M1 [28]. The M1 isoform of AQP4 seems to be important for NMO, since it is preferentially expressed in the optic nerve and spinal cord, where NMO lesions are usually found [29]. However, in another study, the M23 isoform has been proposed as a major target of anti-aquaporin-4 antibodies [15]. In this report an OAP-related, quaternary structure epitope, was identified on the surface of living cells selectively transfected to express M23 isoform. Since native aquaporin-4 assemblies as heterotetramers of M1 and M23 Splice variants forming intermediate size OAPs, the artificial enlargement of the OAPs caused by the over-expression of M23 isoform could enhance the ability of autoantibody binding due to their potential to allow the bivalent binding of autoantibodies. Although this can be an advantage for the diagnostic detection of anti-AQP4 antibodies, it does not imply that all NMO autoantibodies bind only M23.

All 3 B-cell epitopes identified in our study were surprisingly located in the intracellular domains of the molecule. The mechanisms of recognition of intracellular epitopes are more complex, and to a great extend unknown. Many autoantibodies directed against intracellular antigens, including anti-U1RNP, anti-dsDNA, anti-Ro/SSA, anti-La/SSB, anti-Hu and others, have the potential to penetrate cells [30–33], in vivo [34,35]. Alternatively, the priming of the autoimmune response can follow apoptosis and release of intracellular epitopes or internalization. In the case of NMO, the polyclonal response we describe could be following astrocytic damage. A recent study was able to pinpoint CD4+ T-cell epitopes of the AQP4 protein in its intracellular domains (in C57BL/6 and SJL/J mice) [36], suggesting that T-cell assistance may be also necessary to mount an efficient immune response against intracellular fragments of aquaporin. In contrast to our finding, Hinson et al. [10], based on the observation that anti-AQP4 antibodies bind to the surface of AQP4-transfected cells in the absence of conditions allowing membrane permeability, proposed that the epitopes are located in the extracellular regions of the protein. Several other studies have also focused only on the extracellular regions rather than the intracellular domains of aquaporin-4. Tani et al. [16], constructed amino acid substitution mutants in the extracellular domains of mouse M23-AQP4 and after comparisons of immunostaining intensity between human wild-form, mouse and rat AQP4, suggested that the third extracellular loop (E-loop) of AQP4 is an major epitope for the AQP4 antibodies. A search of protein databases led to the detection of a 73% sequence similarity between AQP4 [aa257–271] and a 15-mer peptide from TAX1BP1 protein. TAX1BP1 is a human protein involved in Human T-cell Leukemia Virus type 1 (HTLV-1) replication [37,38]. HTLV-1 is the causative agent of T-cell lymphoma and not in the M23 isoform of the protein, suggesting that the M1 isoform exhibits definite antigenic sites. This finding agrees with Marnetto et al. [17] who showed, using western blot, that the major target of anti-aquaporin-4 antibodies in NMO is the linear AQP4-M1 isoform. Our observation also supports the recent finding of two different missense allelic mutations at Arg19 (R19I and R19T), which enhance susceptibility to NMO and are located within the first 22 residues of the N-terminus of the protein, unique to the AQP4-M1 [28]. The M1 isoform of AQP4 seems to be important for NMO, since it is preferentially expressed in the optic nerve and spinal cord, where NMO lesions are usually found [29]. However, in another study, the M23 isoform has been proposed as a major target of anti-aquaporin-4 antibodies [15]. In this report an OAP-related, quaternary structure epitope, was identified on the surface of living cells selectively transfected to express M23 isoform. Since native aquaporin-4 assemblies as heterotetramers of M1 and M23 Splice variants forming intermediate size OAPs, the artificial enlargement of the OAPs caused by the over-expression of M23 isoform could enhance the ability of autoantibody binding due to their potential to allow the bivalent binding of autoantibodies. Although this can be an advantage for the diagnostic detection of anti-AQP4 antibodies, it does not imply that all NMO autoantibodies bind only M23.

In our study, NMO positive sera were examined for their reactivity against the two homologous 15-mer peptides (derived from AQP4 and TAX1BP1, respectively), revealing a high concordance of positivity. The specificity of the reaction as evaluated by the ability of the 2 peptides to cross-inhibit one another led to high inhibition rates for both peptides. Whether the cross-reactivity of anti-AQP4 antibodies with domains of the TAX1BP1 protein suggests a potential link between NMO and neurotropic retroviruses or it is an epiphemomenon, remains to be determined.

In summary, this is the first B-cell epitope mapping of the autoantigenic AQP4. A proportion of anti-AQP4 antibodies target certain linear epitopes, located in the intracellular domains of the molecule. Our study does not address the conceivable pathogenic role of these specific antibodies but rather raises important questions about the generation of the immune response. One of the 3 epitopes identified in our study, presents high similarity with the human TAX1BP1 protein, which is involved in the pathogenesis of HAM/TSP, a disease that shares common clinical features with neuromyelitis optica. Future studies in our laboratory aim to determine the pathogenic relevance of these findings.

References


