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Enolase and Arrestin are Novel Nonmyelin Autoantigens in Multiple Sclerosis

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Abstract

Introduction—Although myelin autoimmunity is known to be a major factor in the pathogenesis of multiple sclerosis (MS), the role of nonmyelin antigens is less clear. Given the complexity of this disease, it is possible that autoimmunity against nonmyelin antigens also has a pathogenic role. Autoantibodies against enolase and arrestin have previously been reported in MS patients. The T-cell response to these antigens, however, has not been established.

Methods—Thirty-five patients with MS were recruited, along with thirty-five healthy controls. T-cell proliferative responses against non-neuronal enolase, neuron-specific enolase (NSE), retinal arrestin, β -arrestin, and myelin basic protein were determined.

Results—MS patients had a greater prevalence of positive T-cell proliferative responses to NSE, retinal arrestin, and β -arrestin than healthy controls ($p < 0.0001$). The proliferative response against NSE, retinal arrestin, and β -arrestin correlated with the response against myelin basic protein ($p \leq 0.004$). Furthermore, the proliferative response against retinal arrestin was correlated to β -arrestin ($p < 0.0001$), whereas there was no such correlation between non-neuronal enolase and NSE ($p = 0.23$).

Discussion—There is accumulating evidence to suggest that the pathogenesis of MS involves more than just myelin autoimmunity/destruction. Autoimmunity against nonmyelin antigens may be a component of this myriad of immunopathological events. NSE, retinal arrestin, and β -arrestin are novel nonmyelin autoantigens that deserve further investigation in this respect. Autoimmunity against these antigens may be linked to neurodegeneration, defective remyelination, and predisposition to uveitis in multiple sclerosis. Further investigation of the role of these antigens in MS is warranted.

Keywords

Enolase; arrestin; multiple sclerosis (MS); T lymphocytes; autoimmunity

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Introduction

Multiple sclerosis (MS) has long been regarded as an autoimmune demyelinating disorder of the central nervous system (CNS) mediated by T-cells reactive for myelin proteins. Recently, the pathogenic role of myelin autoantibodies has been appreciated as well (1). Several lines of evidence highlight the importance of myelin autoimmunity and T-cells in the pathogenesis of MS. Studies using the well-accepted model of MS, experimental autoimmune encephalomyelitis (EAE), which can be actively induced by immunization with myelin components, have shown that EAE can be passively transferred by activated myelin-specific T-cells (2). Furthermore, T-cell reactivity against various myelin antigens (myelin basic protein, proteolipid protein, and myelin oligodendrocyte glycoprotein) has been demonstrated in MS patients (3). While the role of myelin autoantigens in the pathogenesis of MS is well researched and well established, the potential role of nonmyelin CNS antigens is less researched and less understood.

Transaldolase (TAL) is a key enzyme in the pentose phosphate pathway. High-affinity autoantibodies to recombinant TAL have been detected in serum (25/87) and cerebrospinal fluid (15/20) of patients with MS, while TAL antibodies were absent in 145 normal individuals and patients with other autoimmune and neurological diseases (4). Increased proliferative T-cell responses to recombinant TAL has been reported in MS patients, and this response has been shown to be more pronounced than that obtained by stimulation with MBP (4,5). Enolase is another metabolic enzyme which is potentially interesting in the context of MS. Antibodies against non-neuronal enolase (NNE) have been described in various autoimmune diseases, where they are believed to have a pathological role (6,7). Autoantibodies against NNE have also been implicated as a cause of autoimmune retinopathy (8–10). We, and others have reported the presence of anti-NNE antibodies in association with retinopathy in MS patients (11,12). Furthermore, we have reported an increased prevalence of enolase autoantibodies in MS patients compared to healthy controls (13). Neuron-specific enolase (NSE) is the enolase isoform found in the CNS. Although cerebrospinal fluid (CSF) levels of NSE in MS patients have been investigated as a potential biomarker in MS, no clear difference in the level of this protein has been observed in MS patients compared to healthy controls (14,15). To date, the T-cell response to NNE/NSE in MS patients has not been investigated.

S100 β is a calcium-binding protein that is expressed predominantly in astrocytes, but can additionally be found in other cells such as Muller cells of the retina (16–18). This protein has been found in CSF of 13 out of 18 patients with MS, whereas it was undetectable in any of the 11 control patients (19). CSF studies have also found that there is a significant trend for increasing S100 β levels from primary progressive (PP) to secondary progressive (SP) to relapsing remitting (RR) MS, and that S100 β was significantly higher in RR MS than in control patients, demonstrating that S100 β is a good marker for the relapsing phase of the disease (20). S100 β immunization and S100 β -specific T-cell adoptive transfer can induce EAE and uveoretinitis in the Lewis rat, demonstrating that nonmyelin antigens can have an immunopathogenic role in inflammatory CNS disease (21,22). Analogous to S100 β , arrestin is also a calcium-binding protein that is found in the brain (β -arrestin) and retina (23–25). It is well known that MS is associated with intermediate uveitis (26–28) and arrestin can be used to induce experimental autoimmune uveoretinitis (EAU) (29–31). Autoantibodies to both brain and retina arrestin have been reported in MS patients (11,32,33), although the T-cell proliferative response to this antigen has not been reported.

The purpose of this study was to further explore T-cell autoreactivity against nonmyelin CNS antigens in MS. Based on the reasoning above, we chose to study both enolase and arrestin in this context. T-cell proliferation against different isoforms of these proteins was determined

and compared to myelin basic protein (MBP), which is the most extensively studied candidate autoantigen in MS (3).

Methods

Patients and Controls

Approval for this study was obtained from the Research Ethics Board, St. Michael's Hospital, Toronto, Canada. Informed consent was obtained from all patients and controls. Thirty-five patients with clinically definite MS (CDMS) were recruited from the ambulatory MS clinic. All patients were newly diagnosed and were not taking any immunomodulatory therapy. For controls, we recruited 35 healthy age- and sex-matched volunteers. Expanded Disability Status Scale (EDSS) scores and classification of MS (RR, SP, or PP) was performed by a neurologist specializing in MS (POC). A summary of the patient and control characteristics is displayed in Table I. All of the MS patients had EDSS scores less than 4.

Antigens/Reagents

NNE purified from human brain was commercially purchased (HyTest, Turku, Finland). Human recombinant NSE was a gift from Dr. P. Farrell (Nanogen, Toronto, Canada). Human recombinant retinal arrestin was prepared as previously described (34). β -arrestin purified from bovine brain was a gift from Dr. J. Benovic (Thomas Jefferson University, Philadelphia, USA). MBP purified from human brain was a gift from Dr. M. Moscarello (Hospital for Sick Children, Toronto, Canada). Phytohemagglutinin (PHA) was commercially purchased (Calbiochem, San Diego, USA).

T-cell Proliferation Assays

The T cell assays used in this study have been extensively used and validated in previous studies (35–37). Peripheral blood mononuclear cells (PBMCs) from patients and controls were purified on Ficoll-Paque™ PLUS gradients (GE Healthcare, Uppsala, Sweden) and cultured in 96-well flat-bottom MICROTTEST™ plates (Becton Dickinson, Franklin Lakes, USA) at a density of 10^5 cells/well (after trypan blue exclusion) in the presence of various amounts of test antigen (0.01–5 μ g). The medium used was Serum-free and Protein-free Hybridoma Medium (Sigma, St. Louis, USA) supplemented with human recombinant IL-2 (Roche Applied Science, Indianapolis, USA). Cells were grown in an incubator (37°C/5% CO₂) for 6 days, after which 1 μ Ci³H-thymidine (6.7 Ci/mmol) was added. Following an overnight incubation, cells were harvested and counted using a Beckman Coulter™ LS 6500 scintillation counter. Data are represented as stimulation index (SI), which is defined as the counts per minute (cpm) obtained from PBMCs incubated with test antigen divided by the cpm of PBMCs alone. All experiments were done in duplicate, and personnel performing the experiments were blind to the identity of the samples.

Data Analysis and Statistics

Antigen dose-response curves were produced by log transforming the antigen dose (*x*-axis) and normalizing the SI values (*y*-axis) to define 0% and 100% as the smallest and highest values, respectively. Fischer's exact test was used to compare SI's between patients and controls, with SI's ≥ 1.5 representing a positive response and SI's < 1.5 representing a negative response. Because of multiple comparisons, statistical significance was set at 1%. Linear regression was used to compare the SI's of various test antigens with each other.

Results

Dose-response curves (Fig. 1) of tested antigens at various doses demonstrated that at doses including and exceeding 1 μ g, lymphocytes from healthy controls began showing proliferative responses. Based on the dose-response curve, we chose 0.1 μ g as our antigen dose for further analysis in order to minimize the effects of nonspecific interactions and/or endotoxin in the preparations. The results of our T-cell proliferation assays are summarized in Fig. 2. T-cell proliferative responses to the mitogen PHA in patients and controls were similar, indicating a similar proliferative capacity in both samples. The prevalence of positive responses against MBP, NSE, retinal arrestin, and β -arrestin was significantly higher ($p < 0.0001$) in MS patients and controls. The prevalence of positive responses against NNE, however, did not differ significantly between MS patients and controls ($p = 0.11$).

When T-cell proliferative responses of nonmyelin antigens were compared against those of MBP, we found that the response to NSE, retinal arrestin, and β -arrestin correlated positively with that of MBP ($p \leq 0.004$, Fig. 3). We did not find a correlation between the proliferative response against NNE when compared to MBP ($p = 0.36$). There was a strong correlation between T-cell proliferative responses of retinal arrestin and β -arrestin ($p < 0.0001$, Fig. 4), whereas there was no such correlation between non-neuronal enolase and neuron-specific enolase ($p = 0.23$).

Discussion

We have reported increased prevalence of positive proliferative responses of T-cells from MS patients compared to controls in response to three novel nonmyelin antigens: NSE, retinal arrestin, and β -arrestin. Although there was a higher prevalence of positive proliferative responses against NSE in MS patients compared to that in controls, we did not find any difference in proliferative responses against NNE. Enolase is a glycolytic enzyme (2-phospho-D-glycerate hydrolase), which exists in three isoforms: α -enolase or NNE, which is ubiquitous, β -enolase, which is predominant in muscle, and γ -enolase of NSE, which is found in neurons, paraneuronal cells, and neuroendocrine cells (38,39). Recently, the important contribution of neurodegeneration to the pathogenesis of MS has been appreciated (40,41). It is apparent that inflammatory and neurodegenerative factors are both involved in the pathology of MS (42). Patients with various systemic autoimmune diseases have been reported to have autoantibodies against α -enolase, but cross-reactivity with NSE in these diseases is rare (6). Neurons and glial cells (including oligodendrocytes) are known to express NSE (43,44); so, it is not surprising to see higher autoreactivity against NSE compared to NNE in an autoimmune disease such as MS, which primarily affects the CNS.

Autoimmunity against NSE may be linked to the neurodegenerative component of MS. Examination of NSE levels in the cerebrospinal fluid of MS patients has not been promising in the search for a relevant biomarker of axonal degeneration (14,15). A recent study examined serum levels of S100 β and NSE in MS patients over a five-year period in order to find a marker for disease progression. Interestingly, although no correlation was found for serum S100 β levels, a strong inverse relationship was found between serum NSE levels and disease progression (45). One explanation for this observation is that the lower serum NSE levels in MS patients with a progressive disease course and more severe disability reflect reduced metabolic activity secondary to axonal loss. Our patient cohort contained all newly diagnosed patients, therefore, we can assume that the mean level of disability in our cohort was less than that in the above-mentioned study. If serum NSE levels are a marker of axonal loss, then it is interesting to speculate the significance of T-cell reactivity against NSE. Perhaps NSE autoreactivity is high early in the disease (as we observed) because axonal/glial load is still high, but then the autoreactivity drops off as neuronal and glial mass decreases. A longitudinal

study measuring NSE autoreactivity in MS patients is required, and warranted, to test this hypothesis.

T-cell reactivity against arrestin may explain why MS patients are susceptible to intermediate uveitis. We observed similar T-cell responses to arrestins and MBP in MS patients. Both antigens had a higher prevalence of positive responses in MS patients compared to controls, and the reactivity against these antigens was correlated. MS is associated with uveitis (26–28), and arrestin is a principal autoantigen in uveitis (46,47). The majority of HLA population studies in MS have focused on Caucasians of Northern European descent, where the predisposition to disease has been consistently associated with the HLA-DR15 haplotype (a subtype of HLA-DR2) (48). Intermediate uveitis has also been associated with the HLA-DR15 haplotype (49,50). T-cell responses to the immunodominant epitope of retinal arrestin, peptide M, have been documented in several populations of uveitis patients (51–53). The immunodominant T-cell epitope of MBP has been localized to residues 82–98; this MBP peptide binds with high affinity to the disease-associated HLA-DR2 molecule, and is recognized by HLA-DR2-restricted T-cell clones from MS patients (54). Based on competitive binding studies, it has been demonstrated that important antibody epitopes, MHC and T-cell receptor (TCR) contact residues lie in this region (55). F89, F90, and K91 were key residues for autoantibody binding, while H88, F89, and K91 were primary contact residues for MBP-specific TCRs, and V87 and F90 were MHC anchor residues. When the amino acid sequence of MBP 82–98 is aligned with that of peptide M (Fig. 5), many important similarities are seen. The key residues for antibody, TCR and MHC binding (F89, F90, and K91), are either conserved or replaced with homologous amino acids between these residues. Furthermore, several other amino acids in the flanking regions are conserved or replaced by homologous residues. This similarity in immunodominant peptide sequences is likely one explanation for the observed association between MS and intermediate uveitis.

Autoreactivity against β -arrestin may partly explain the impaired remyelination and delayed oligodendrocyte development observed in MS. β -arrestin is a protein that is vital for signal transduction (56). It has been demonstrated that sonic hedgehog, a protein vital for oligodendrocyte development, is decreased in white matter of MS patients (57). Although the exact molecular mechanism is yet to be determined, it has been suggested that sonic hedgehog signaling may have a role in facilitating remyelination (58). Moreover, dysfunction of the Notch signaling pathway has been implicated in the limited remyelination in MS lesions (59, 60). In this context, it is interesting to note that β -arrestin is involved in both Notch and sonic hedgehog signaling (61–63). Alignment of the amino acids residues of the β -arrestins (25,64) reveals a sequence that is homologous to the immunodominant epitopes of MBP and retinal arrestin (Fig. 5), suggesting that common epitopes may explain the similar T-cell responses to these antigens.

The T-cell response to NNE was not significantly different between patients and controls. In contrast, positive T-cell proliferative responses to NSE were more prevalent in MS patients and were highly correlated with MBP, suggesting that homologous epitopes are present in these molecules. Immunogenic epitopes within human NSE have not been characterized, making the search for a common epitope difficult. The α -subunit of NNE is 336 amino acids residues in length (65), whereas the γ -subunit of NSE is 434 amino acids long (66). The polypeptide sequences of these subunits align together with the first 98 residues of NSE being unique to that molecule. Our observations of contrasting antigenicity between NSE and NNE suggest that the immunodominant epitope of NSE lies within the first 98 amino acid residues of this molecule.

One plausible explanation for the evolution of antigenic diversity in MS may be epitope spreading, whereby the target of an immune response extends from the intended antigen to

other epitopes present on the cell involved in the primary response. Epitope spreading, both within the same myelin molecule (intramolecular spread) as well as to different myelin molecules (intermolecular spread), has been documented well in several EAE models (67–71). Furthermore, spreading of immunodominant determinants has been reported in patients with uveitis (72). In patients with isolated monosymptomatic demyelinating syndromes (IMDS), a group of distinct clinical disorders with variable rates of progression to MS, differential autoreactive patterns to myelin peptides with epitope spreading have been demonstrated within patient subpopulations (73). The role of epitope spreading in CDMS, however, has not been established. Furthermore, epitope spreading between myelin and nonmyelin antigens has not been documented in EAE or MS. Based on our observations, we feel that epitope spreading between myelin and nonmyelin antigens in MS deserves further investigation.

Although once thought of almost exclusively as an autoimmune inflammatory disease against myelin antigens, it is becoming increasingly apparent that the pathogenesis of MS is far more complex. Recent evidence has shown that early axonal degeneration, oligodendrocyte apoptosis, impairment of remyelination, vascular changes, and instability of myelin proteins are all involved in the disease process (14,42,74,75). Given this high degree of complexity, it is plausible that antigens other than those derived from myelin are involved in the immunopathology of MS. Autoimmunity against membrane-bound receptors and/or intracellular proteins involved in metabolism and signal transduction may be involved in a myriad of events which occur in the evolution of this disease. We have identified two novel antigens, enolase and arrestin, which are recognized as autoantigens by T-cells in MS patients. The T-lymphocyte proliferation against these nonmyelin antigens correlated with the response against MBP, a myelin protein that has a clear and established role in the pathogenesis of MS. Based on our observations and evidence in the literature, we believe that these novel nonmyelin antigens may indeed be contributing to the pathogenesis of MS and deserve further investigation.

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Fig. 1.

Dose-response curves of various antigens in MS patients and controls. At doses including and exceeding the third data point ($1 \mu\text{g}$), lymphocytes from healthy controls began showing proliferative responses. Based on the dose-response curve, we chose the second dose point ($0.1 \mu\text{g}$) as our antigen dose for further analysis. NNE = non-neuronal enolase, NSE = neuron-specific enolase, Arr = retinal arrestin, β -Arr = β -arrestin, and MBP = myelin basic protein.

Fig. 2.

T-cell proliferative responses to various test antigens. Results are expressed as SI.

Phytohemagglutinin (PHA) response was determined at $5 \mu\text{g}$, and all antigens were tested at $0.1 \mu\text{g}$. Horizontal dashed line represents $\text{SI} \geq 1.5$, the cutoff for a positive response. NNE = non-neuronal enolase, NSE = neuron-specific enolase; Arr = retinal arrestin, β -Arr = β -arrestin, and MBP = myelin basic protein; * = $p < 0.0001$.

Fig. 3. Correlation of T-cell proliferative responses to various test antigens ($0.1 \mu\text{g}$). Stimulation indexes (SI) for non-neuronal enolase (NNE), neuron-specific enolase (NSE), retinal arrestin (Arr), and β -arrestin (β -Arr) were compared to myelin basic protein (MBP). Pearson correlation coefficient (r) is shown, along with its p value.

Fig. 4. Correlation of T-cell proliferative responses to various test antigens ($0.1 \mu\text{g}$). (A) Stimulation indexes (SI) for non-neuronal enolase (NNE) were compared against neuron-specific enolase (NSE). (B) Stimulation indexes (SI) for retinal arrestin (Arr) were correlated with β -arrestin (β -Arr). Pearson correlation coefficient (r) is shown, along with its p value.

Peptide	Amino Acid Sequence																		
Human MBP (82-98)	D	E	N	-	P	V	<u>V</u>	<u>H</u>	F	F	K	N	I	V	T	P	R	T	-
Human Peptide M	D	T	N	L	A	S	S	T	I	I	K	E	G	I	D	-	R	T	V
Human β_1 -Arr (297-314)	D	T	N	L	A	S	S	T	L	L	R	E	G	A	N	-	R	E	I
Human β_2 -Arr (298-315)	D	T	N	L	A	S	S	T	I	V	K	E	G	A	N	-	K	E	V

Fig. 5.

Amino acid sequences of immunodominant peptides in MBP and arrestins. The sequence of MBP 82–98 has been aligned with similar sequences in human arrestins (including peptide M of retinal arrestin). Identical amino acids are highlighted *green*, whereas homologous amino acids are highlighted in *yellow*. A *dashed line* indicates an empty space (no amino acid). Amino acids in *bold* are key residues for autoantibody binding, while *underlined* amino acids are important for binding to T-cell receptors and MHC molecules.

Table I

Characteristics of Patients and Controls

	Mean age	Sex ratio (F:M)	MS type (RR, SP, PP)
Patients	42 ± 10	1.7:1	(77%, 20%, 3%)
Controls	39 ± 13	2.2:1	—

Note. Age is represented as $\pm$ SD.