

Serum IgE reactive against small myelin protein-derived peptides is increased in multiple sclerosis patients

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Abstract

Though independent findings suggest roles for the allergic arm of the immune system and myelin-reactive antibodies in MS, myelin-reactive IgE has not been investigated. We have developed a radioimmunoassay that measures reactive IgE, IgG and IgA against short (5–6-mers) myelin protein-derived peptides bearing little to no sequence identity with other human proteins, and which might therefore be targets of a CNS-specific autoimmune attack. Here we show that, irrespective of clinical subtype, MS patients' sera are characterized by a higher frequency of measurable IgE against the peptides. Moreover, in controls with measurable IgE reactive against test peptides, IgG or IgA reactive with the same peptide epitopes is almost always present in vastly greater quantities, whereas in MS subjects peptide-reactive IgA or IgG is often undetectable. The sensitivity of the full assay, when considering *overall positive* a serum sample that has detectable autoreactive IgE without other competing Igs, is 69% (S.E.: 5%), with a specificity of 87% (S.E.: 9%). We speculate that IgE reactive against CNS target antigens may have both diagnostic and pathogenic significance, particularly if other peptide-specific, potentially blocking Igs are absent.

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1. Introduction

While its etiology(ies) is unknown, MS is clearly an immune-mediated disease influenced by genetic and environmental factors (Dyment et al., 2004; Granieri, 2000; Oksenberg and Hauser, 2005), and the autoimmune process likely involves both cellular and humoral arms. It is generally assumed that the disease trigger might be an infectious agent that instigates an appropriate immune attack (Granieri et al., 2001), but that through the process of molecular mimicry (Wekerle and Hohlfeld, 2003), an autoimmune attack against a CNS target antigen ensues. Potential myelin or myelin associated target antigens include but are not limited to: myelin-oligodendrocyte

glycoprotein (MOG) (Gardinier et al., 1992), myelin basic protein (MBP) (Zamvil et al., 1986), proteolipid protein (PLP) (Greer et al., 1997), oligodendrocyte-specific protein (OSP) (Bronstein et al., 1999) and oligodendrocyte-myelin glycoprotein (OMgp) (Mikol and Stefansson, 1988). In animal models, a number of candidate myelin and neural proteins have been shown to trigger inflammatory demyelination (Schmidt, 1999). Though EAE is generally considered a T-cell-mediated disease, antibody-mediated myelin destruction is thought to occur in both EAE and MS (Genain and Hauser, 1996; Genain et al., 1995). Using gold-conjugated myelin peptides, myelin peptide-specific antibodies have been demonstrated *in situ* in MS as well as experimental autoimmune (formerly allergic) encephalomyelitis (EAE) lesions (Genain et al., 1999; Raine et al., 1999), strongly suggesting that antibody-mediated myelin destruction occurs in MS.

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The potential biomarker roles of serum IgG and IgM reactive with myelin proteins has gained momentum recently (Berger et al., 2003; Gaertner et al., 2004; Lampasona et al., 2004), but testing has not achieved diagnostic capability. The reason for discrepancy of results between the various studies appears to be due to the different assay systems employed (e.g., liquid vs. solid phase, nated vs. denatured source antigen). In studies published to date, individual epitopes were not identified, Ig isotypes other than IgG and IgM were not examined, and antibodies against potential target antigens other than MOG and MBP were not tested. Nonetheless, these studies have sparked interest regarding the diagnostic and prognostic potential of serum Ig reactive to CNS antigens, in particular MOG.

Autoreactive IgE is implicitly harmful in the proper context of antigen and supporting immune cells. Supportive data exist both clinically and experimentally for involvement of the allergic arm of the immune system in EAE and MS (Lock et al., 2002; Pedotti et al., 2003, 2001), and anti-MBP IgE has been detected in EAE mice (Moore et al., 1974; Toms et al., 1990). IgE reactive to CNS antigen has not been carefully measured in MS patients, however, which at least in part reflects the lack of a sensitive assay to measure this low abundance isotype. Here we provide preliminary evidence that, regardless of clinical subtype of MS, serial measurement of IgE and other Ig isotypes reactive against myelin protein derived peptides has diagnostic potential. We speculate that autoreactive IgE may be pathogenic and, as such, we explore how an IgE-mediated mechanism might fit with our current understanding of the immunopathogenesis of MS.

2. Materials and methods

2.1. MS patients and controls

This study was approved by the University of Michigan Institutional Review Board. Patients with relapsing or progressive MS, ages 18–65, were enrolled from the MS Clinic pool without selection bias. MS patients were excluded if they had a known autoimmune or neurological disorder other than MS; controls were excluded if they had a known autoimmune or neurological disorder or history of MS in any family member. In this cross-sectional study, sera from 26 individuals with MS were analyzed: 5 secondary progressive (SP), 5 primary progressive (PP) and 16 relapsing-remitting (RR), including 2 with clinically isolated demyelinating event; one (MS1) without demonstrated MRI change and a second (MS2) with MRI change over time consistent with RRMS according to accepted diagnostic criteria (McDonald et al., 2001). In most cases, patients were on interferon-beta (18) or glatiramer acetate (3), though some (5) were on no disease-modifying treatment; 3 patients had received mitoxantrone within the past year (MS12: cumulative 36 mg/m², last infusion 10 months earlier; MS19: 24 mg/m², ongoing treatment with last infusion 3 months earlier; MS21: 72 mg/m², last infusion 7 months earlier). Healthy controls were

drawn from spouses or friends of patients and from volunteers in the University of Michigan Neurology Clinic. In the case of MS patients, disease duration and EDSS at the time of serum collection were recorded. Tubes containing MS and control sera were labeled using a random number system.

2.2. Development of a panel of short, unique myelin protein-derived peptides (SUMPPs)

Based on our early, unpublished observations testing serum IgE reactivity against various sized peptides derived from known primary structures of myelin proteins, a major modification was made: the introduction of a smaller number of SUMPPs, 5 or 6 amino acids in length. This change was based on observations that IgE reactivity against synthetic peptides derived from myelin proteins appeared to be more disease-specific when employing small peptides (5–6 contiguous amino acids) as compared to longer representative peptides. The use of small peptides is supported by findings that the minimum size of a linear peptide required for antibody binding is ~5–6 amino acids in length (Hinterhuber et al., 2005; Kabat, 1966; Wilson et al., 1984).

Peptide selection was based on the hypothesis that, if a peptide epitope is likely to be of significance diagnostically or pathologically, its sequence is likely to be relatively unique, so as to present a target for a tissue-specific autoimmune attack (Fig. 1), which is supported by our early work with the relatively unique peptide sequence ADARM, derived from PLP. Eight SUMPPs (5–6-mers) derived from three myelin proteins were selected for analysis: AAMEL, HSYQE, QAPEY and VTLRI from the extracellular portion of MOG; HRTFE and KTGQFL from the cytoplasmic region of MOG; AHRET from OSP (cytoplasmic); and ADARM from PLP (extracellular). When comparing the pentameric or hexameric sequences of the eight chosen peptides to all human proteins, they are unique to their parent myelin surface, except in a small number of cases where they are situated in the interior of a protein and therefore apt to be less accessible for antibody binding. Biotinylated peptides were synthesized with an 8-amino-3,6-dicyclohexylidene-3-methylbutyl group linker by Mimotopes, Ltd. (Melbourne, Australia). Peptides were synthesized with an N-terminal biotin and a polyethyleneglycol (PEG) spacer (biotin-PEG-peptide) to facilitate their solubility and availability for antibody binding, insofar as most of the relevant peptides are relatively hydrophobic in their free state.

2.3. Materials and assay preparation

Testing of MS and control sera for IgE reactivity against individual SUMPPs was carried out blindly. Serum IgE was purified from neat serum (see below), as other Igs might otherwise compete with binding, given that IgE is several orders of magnitude less concentrated. Paper discs obtained from Schleicher and Schuell (Keene, NH) were cyanogen bromide-activated (Ceska et al., 1972), and then separately

PLP	tRNA guanine-N2-N2 methyltransferase (BAA91031)	PLP	calcium-response factor (AAL62330)	leupaxin (CAG38768)	mucin 5 (CAC83675)	N-acetylated alpha-linked acidic dipeptidase-like 1 (NP_005459)	sodium channel 1 (AAC50432)	titin (CAD12456)	OMgp	RGS domain-less RGS12TS isoform 2 (AAL69960)	zinc finger DHHC domain containing 19 protein (BC022078)	3 beta-hydroxy-delta 5-C27-steroid oxidoreductase (AAH04929)	recombining binding protein suppressor of hairless, isoform 4 (AAH64976)	transmembrane protein 16C (CAC32454)	C3 and PZP-like alpha-2-macroglobulin domain containing 8 (AAM50084)	skeletal myosin light chain kinase (CAC10006)	DNA helicase HEL308 (AAL85274)	RGS12 protein (AAI06945)	C3 and PZP-like alpha-2-macroglobulin domain containing 8 (AAM50084)	RIC3 protein (AAP92162)
i	v	s	n	e	s	y	m	y	d	d	t	t	s	s	e	g	g	d	e	e
g	q	i	d	s	t	t	v	k	t	c	h	t	w	i	e	i	a	c	e	g
s	p	a	g	p	p	y	k	v	t	v	l	f	t	t	e	q	v	v	e	l
l	s	f	e	p	q	d	i	a	f	q	p	t	i	l	r	n	a	q	r	g
c	q	p	e	p	t	r	p	t	t	l	f	v	i	i	g	p	s	l	g	i
a	a	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s
d	d	k	k	k	k	k	k	k	t	t	t	t	t	t	t	t	t	t	t	t
a	a	t	t	t	t	t	t	t	d	d	d	d	d	d	d	d	d	d	d	d
r	r	s	s	s	s	s	s	s	k	k	k	k	k	k	k	k	k	k	k	k
m	m	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
y	l	s	q	a	a	r	k	k	f	g	i	k	e	e	r	p	g	r	y	
g	m	i	v	a	t	i	y	l	v	v	a	a	y	q	h	k	v	h	t	
v	y	g	f	q	s	y	l	k	p	v	i	q	t	v	f	g	v	f	g	
l	q	s	e	l	s	p	e	v	y	a	v	r	f	n	l	p	a	l	s	
p	h	l	h	d	t	t	k	e	p	e	v	h	y	t	e	t	e	e	m	

Fig. 1. ADARM as an example of a small, unique peptide sequence. Homology of peptide sequences within PLP (ADARM and SKTSA) and OMgp (STDKA) reveals very little sequence identity between ADARM and other human proteins, but for SKTSA and STDKA, sequences are shared by a number of proteins. The ADARM sequence is only found in an intracellular protein, tRNA guanine-N2-N2 methyltransferase. The SKTSA and STDKA sequences are more common among human proteins. Proteins containing the sequence of interest are indicated with flanking sequences (accession numbers in parentheses).

conjugated with neutravidin (NA; Pierce, Rockford, IL), lysine-PEG-biotin (Mimotopes, Clayton, Australia), or affinity-purified, goat polyclonal, anti-human IgE antibody (Vector Laboratories, Burlingame, CA).

2.4. Peptide-specific IgE measurement

In order to measure SUMPP-specific serum IgE, care was taken to avoid potential interference from specific non-IgE antibodies and nonspecific IgE antibodies. Toward that end, individual serum samples (1 ml each) were incubated for 20 h with anti-IgE conjugated discs to adsorb all IgE in the sample. The paper discs were then washed with pH 7.4 phosphate buffered saline (PBS), and captured IgE was eluted with 0.5 ml acetic acid, pH 2.2 and immediately neutralized with an equal volume of 1.0 M dibasic sodium phosphate containing 1 mg/ml globulin-free human serum albumin (Sigma-Aldrich, St. Louis, MO) and 0.5 mg/ml sodium azide. To each eluted, buffered IgE sample, two paper discs containing conjugated NA were added per ml of IgE solution in order to adsorb anti-avidin IgE.

Each biotinylated peptide was diluted in PBS plus 0.05% Tween-20 (PBST) and mixed with NA in PBST (2:1 molar ratio); as control, NA alone was added to PBST. 5 ng of each peptide/NA complex or 4.7 ng NA alone was separately added to 0.5 ml IgE solution, followed by gentle vortexing and incu-

bation at 37 °C for 2 h; 100 µl of each mixture was then added, in quadruplicate, to paper discs containing covalently coupled, projecting lysine-PEG-biotin. Paper discs were washed in PBST and 50 µl ¹²⁵I-labeled, polyclonal goat anti-IgE (40,000 counts/min) diluted in 2% heat treated horse serum was added and incubated for 20 h. 25 IU IgE control sera (Hycor Biomedical, Inc., Garden Grove, CA) plus 50 µl ¹²⁵I-labeled, polyclonal goat anti-IgE was used as a positive control for each run and served to standardize counts from day to day. Discs were washed and counted for 10 min each in a gamma counter (Packard, Meriden, CT). As four discs were counted for each SUMPP analyzed per subject, high and low counts for the SUMPP and NA discs were discarded and the middle two signal values were averaged. Finally, the NA signal for each test subject was subtracted from each corresponding SUMPP signal value to yield IgE anti-SUMPP counts for each serum sample. For individuals whose IgE counts were >0 for a given peptide, serum was tested for IgG reactivity against the same peptide (see below).

2.5. Peptide-specific IgG and IgA measurement

Biotinylated SUMPPs (50 ng) in 1 ml PBST were separately added to 4 paper discs covalently coupled to NA (4 µg/disc) and incubated overnight at 4 °C. The discs were washed, and 100 µl

neat serum from test subjects who were IgE-positive against one or more SUMPP was individually applied to specific SUMPP discs and non-peptide NA discs, each in quadruplicate, for 15 min. The shorter incubation time was used in view of the much greater concentrations of IgG and IgA in serum relative to IgE, and the fact that Ig binding to peptide is exponential over time (e.g., 10 net counts at 15 min = ~40,000 counts at 2.2 h). The serum samples were then quickly aspirated and the discs washed. Employing the basic procedure used for specific IgE measurement, ¹²⁵I-labeled, affinity-purified, goat anti-IgG was applied to discs sets, followed by incubation at 37 °C for 20 h, aspiration, washing and counting as for specific IgE. The peptide-specific, middle two peptide disc signal values were averaged as were the middle two NA disc values. The NA signal for each test subject was subtracted from each corresponding peptide signal value to yield net SUMPP-specific IgG counts. For subjects without detectable IgG reactive against a SUMPP, specific IgA was measured in a similar fashion using ¹²⁵I-labeled, affinity-purified, goat anti-IgA in place of anti-IgG.

The present assay techniques allow quantitative detection of SUMPP-specific serum IgG, IgA and IgE, the latter of which is the least abundant Ig isotype (<240 ng/ml range). For comparative purposes, counts are depicted for SUMPP-specific Ig. In general, for the same number of counts, the quantity of IgA or IgG is ~16,000× that of IgE (e.g., 30 counts IgE = 4.4 pg/ml serum, whereas for IgA and IgG, 30 counts = 70 ng/ml serum). SUMPP-specific antibody measurement was analyzed in terms of: (1) reactive IgE levels alone and (2) reactive IgE, IgG and IgA. In the latter case, our algorithm assigns *overall negative* results to subjects having no SUMPP-specific IgE or measurable amounts of SUMPP-specific IgE but potentially blocking IgG or IgA (i.e., the latter being present at >640-fold greater concentrations). *Overall positive* results are assigned to subjects having SUMPP-specific IgE but no detectable, potentially blocking IgG or IgA against the same SUMPP.

3. Results

3.1. Practical and theoretical bases for peptide selection

As discussed in Section 2, based on preliminary testing (data not shown) for IgE reactivity against peptides of varying lengths from MOG, OMgp, OSP and PLP, we shifted our focus to testing of SUMPPs, peptides 5 or 6 amino acids in length and of rather unique sequence. Our prior work included several pentameric peptides: ADARM and SKTSA from PLP and STDKA from OMgp. Of these, only ADARM appeared promising, as serum IgE against this peptide could be detected with higher frequency among MS patients compared to controls. When these three peptide sequences are searched (<http://www.ncbi.nlm.nih.gov>) across all human proteins, though identical in size, ADARM emerges as being relatively unique in that it is only found on the outer surface of a single protein (PLP), whereas SKTSA and STDKA share sequence identity with a variety of human proteins, and in many cases

the sequence is surface exposed (Fig. 1). Based on these observations, we theorized that when a given linear peptide sequence is common to many different endogenous proteins and is accessible (surface exposed), a directed autoimmune response is unlikely. We therefore developed a library of new peptides in addition to ADARM, with requirements being small size (5–6 contiguous amino acids) and little to no homology with other endogenous proteins (no sequence identity to the extracellular portions of other proteins), which provides a theoretical basis for a CNS-specific autoimmune attack.

3.2. Measurement of serum IgE against SUMPPs

MS patient and control sera were analyzed blindly for measurable IgE against the eight SUMPPs, as described in Section 2. Demographics for the 41 subjects tested are summarized in Table 1. There is no significant difference between MS patients and controls in terms of age, sex or % female. Using the eight SUMPPs as bait, reactive IgE was detectable in many MS and some control sera, as shown in Fig. 2 (actual counts listed for each subject if >0). For several SUMPPs, MS subjects have significantly more reactive IgE, whereas for other SUMPPs, there is no significant difference between MS subjects and controls (Fig. 3). In addition to HRFTE and KTGQFL, for which significantly more MS patients have specific IgE, regardless of magnitude ($p=0.0083$ and 0.0061 , respectively), ADARM (PLP) shows a trend toward exhibiting more reactive IgE in MS subjects ($p=0.0516$).

From this work, it is evident that individuals often exhibit IgE against a number of peptides. Without considering the magnitude of IgE positivity against the SUMPPs, among 26 MS subjects, a total of 128 peptides show some IgE reactivity (mean 4.9 per subject), compared to 59 among the 15 controls (mean 3.9 per subject). In order to explore which peptides might be most predictive of MS, a logistic regression analysis was performed for all 41 subjects using as variables: individual SUMPP-specific IgE positivity or negativity, individual SUMPP-specific IgE levels, age and sex. Using the method of stepwise selection, only two peptides showed a significantly predictive impact: HRFTE and KTGQFL (both contained within MOG) IgE positivity or negativity, with no additional predictive ability due to magnitude of IgE positivity. The model was also assessed for predictive

Table 1
Demographics of MS and control subjects

	<i>n</i>	% Female	Mean age	Mean EDSS	Disease duration
Controls	15	60.0	41.3	NA	NA
All MS	26	61.5	41.3	3.10	9.3
RRMS	16	68.8	35.7	1.72	6.2
SPMS	5	60.0	48.2	5.30	16.4
PPMS	5	40.0	52.2	5.30	11.9

Basic demographics of control and MS groups are shown. No significant differences were seen between controls and MS subjects in terms of age and % female.

		PLP	OSP	MOG	MOG	MOG	MOG	MOG	MOG
		ADARM	AHRET	QAPEY	VTLRI	HSYQE	AAMEL	HRTFE	KTGQFL
Subject									
RRMS	MS1	950	1,044	245	746	37	272	272	466
	MS2	147		3	152	79		433	152
	MS3	1433	572	713	197	530	88	163	985
	MS4	589			705			1020	1188
	MS5	478	362	156		550	1058		537
	MS6			1449		742		426	1397
	MS7			491		476	143	309	241
	MS8	755	150		294		69	188	166
	MS9		4	191	81			523	252
	MS10		75	406	114		37		79
	MS11		164	676			2	3	
	MS12		422	106	519	725		748	
	MS13		13					223	
	MS14			2612	612		420	1077	192
	MS15		437	1051		722	1041	1210	519
	MS16	670	94	1464	379			500	280
SPMS	MS17	1229		90		293	118	954	499
	MS18	237		44	24	60	84	80	94
	MS19			500	200		473		
	MS20	145	26			42	109	90	43
	MS21	1149		1171			378		93
	MS22	49		92			1	100	133
PPMS	MS23						184	276	
	MS24	216	72	252	64		83		
	MS25	198	198	805	418		910	622	
	MS26	14	69	109			9		160
	C1			589			232		
	C2			827			113		
Control Subjects	C3	11		277		3			
	C4		142	469	286	56	161	642	57
	C5	79	133	663	510		115	125	28
	C6	748	318	1815	533	939	515	726	1189
	C7				682		550		
	C8		173	125	427	265	173		
	C9		331	224	256	767	2423		
	C10			138	64		77	3	
	C11			278	201		26		3
	C12			846	10		435		
	C13			171			237		
	C14			408		245	559		
	C15			748				78	

Fig. 2. SUMPP-specific IgE is increased in MS subjects vs. controls. IgE reactive with each SUMPP tested is displayed as radioactive counts, as described in Section 2. Blanks denote no reactivity. Bold numbers reflect IgE counts without detectable IgG or IgA against the same peptide (based on results depicted in Fig. 4); subjects whose number is in bold have IgE without reactive IgG or IgA against ≥ 1 SUMPP. MS subjects more often have measurable IgE against SUMPPs.

accuracy via estimation of an ROC curve using a cross validation technique based on omitting one individual at a time and classifying each omitted individual based on a model fitted to the remaining data. This yielded an estimated sensitivity of 58% (S.E.: 10%) and specificity of 75% (S.E.: 11%). Future work will determine whether certain peptides are more useful diagnostically and in terms of clinical stratification (e.g., associated with relapsing vs. progressive MS), and whether magnitude of autoreactive IgE has clinical relevance.

3.3. Measurement of serum IgG and IgA against SUMPPs

A priori, we were interested in measuring levels of SUMPP-specific IgG and IgA in IgE-positive individuals,

based on the hypothesis that the net balance of reactive isotypes against a target antigen might be associated with disease. Conceptually, autoreactive IgE may be harmful if it is able to bind to its target, with consequent mast cell degranulation. However, if nonharmful Ig of some other isotype is reactive with the same target but is present at much higher concentration, then autoreactive IgE could potentially be blocked. This reasoning forms the basis for our diagnostic algorithm. IgG reactive with SUMPPs was measured in IgE-positive subjects and, if negative, reactive IgA was measured

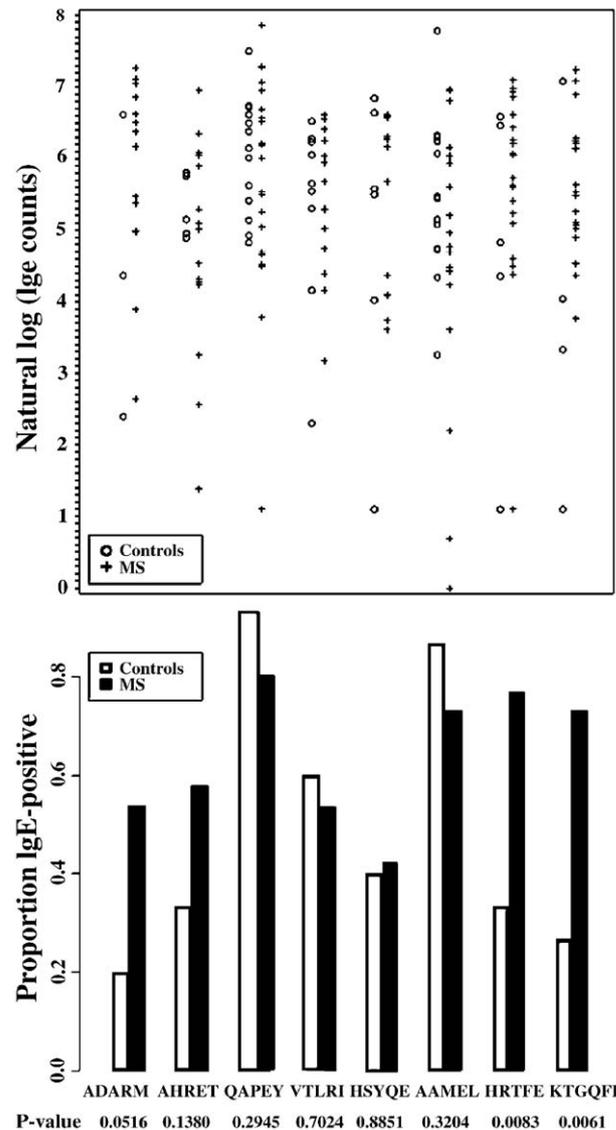


Fig. 3. Distributional summary of SUMPP-specific IgE. For MS and control samples, data from Fig. 2 were compared for each SUMPP. Bar graphs denote the proportion of subjects with measurable (>0) IgE against that peptide. For some peptides, significantly more MS subjects have measurable IgE as compared to controls (*p*-values shown below each peptide). For each peptide, scatter plots of the IgE levels are shown above for subjects with measurable IgE against that peptide (*y*-axis, natural logarithm of IgE level); the distributions of IgE levels for cases and controls are not significantly different for any peptide.

		PLP		OSP		MOG		MOG		MOG		MOG		MOG				
		ADARM		AHRET		QAPEY		VTLRI		HSYQE		AAMEL		HRTFE		KTDQFL		
Subject		IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	
RRMS	MS1	0	0	466		0	1621	1677		1847		2419		614		628		
	MS2	0	0					0	757	0	263		0	438	0	1608		
	MS3	0	0	298		0	1274	436		0	1,263	1424		1323		433		
	MS4			1832								197		2196		1187		
	MS5			2197	0	604	0	680		4581		5149				633		
	MS6					0	0			2438				0	0	148		
	MS7							1222		1987				0	0	0	0	
	MS8	420				1353			1749					0	0	0	114	0
	MS9					828		0	0	1349					172		2173	
	MS10					0	1935	0	1968	510			1548			0	0	
	MS11					1094		912										
	MS12					836		385	587		0	763		0	1245			
	MS13					0	0								207			
	MS14							673	1347				0	496	0	969	790	
	MS15					337		523			0	98	0	98	0	259	0	0
	MS16	0	430	1206		970		0	60					272		0	0	
SPMS	MS17	264				0	0			949		2752		787		693		
	MS18	0	548			177		0	586	911		2122		734		1372		
	MS19					65	783	2298				310						
	MS20	220	873	0	116					0	225	0	0	0	0	0	310	
	MS21	0	0			0	0					0	245			0	0	
PPMS	MS22	2168				0	0							110		0	2008	
	MS23											1599		2405				
	MS24	2152		1999		0	0	307				881						
	MS25			345		261		1711				0	0	824				
	MS26	2690		1070		852										0	0	
Controls	C1					0	886					635						
	C2					1176						67						
	C3	591				1822				1809								
	C4			672		206		954		1755		2433		0	696	1903		
	C5	0	2801	337		0	74	3150				0	250	0	1306	2193		
	C6	0	278	3259		0	1576	3075		0	964	0	0	96	271	2350		
	C7							0	1332			0	2461					
	C8			2284		1388		1343		1507	64	2055						
	C9			2461		0	1824	0	79	0		0	130					
	C10					0	1808	0	865			0	1286					
	C11					983		0	85			1776						
	C12					732		10	859			1569						
	C13					1605						0	1452					
	C14					1342				4523		2797						
	C15					0	0							2602				

Fig. 4. SUMPP-specific IgG, IgA in IgE positive subjects. Subjects whose serum showed IgE reactivity against one or more SUMPP were tested for IgG and/or IgA reactivity, displayed as radioactive counts. IgG was measured first and, if <640× the IgE level against that peptide, IgA reactivity was measured as well. No reactivity is indicated by a 0. If IgG and IgA levels were each <640× the IgE level, that subject was considered overall positive (as shown by side-by-side boxed 0's). While 18/26 MS subjects (69%) have reactive IgE without measurable IgG or IgA against a given peptide (3 with 2 reactive SUMPPs, 2 with 3 reactive SUMPPs), 2/15 controls (13%) have reactive IgE without measurable IgG or IgA against a single peptide.

(Fig. 4). In the overall analysis, IgE reactive subjects who exhibited IgA or IgG at a concentration >640 the IgE concentration against a given peptide were considered overall negative, while those without measurable IgA or

IgG against the same peptide were considered overall positive. Using this algorithm, the estimated sensitivity of the IgE+/IgG-/IgA- assay is 69±5% (18/26 MS subjects positive against >1 peptide), while the estimated specificity

is $87 \pm 9\%$ (2/15 controls positive against 1 peptide each). Of the 128 SUMPP-reactive IgE tallies in the MS group (see Section 3.2), 25 (19.5%) are not associated with IgG or IgA against relevant peptides, compared to 2/59 (3.4%) in the control group (Fig. 4). Neither of the control subjects had “unprotected” IgE against more than one SUMPP, whereas this scenario occurred in 5/18 IgE-positive MS subjects.

A statistical model was considered wherein IgG and IgA negativity was analyzed; subjects were considered *overall positive* if against at least one SUMPP they exhibited measurable (>0 counts) IgE but were IgG and IgA negative. At the same time, measurable IgE against HRTFE and/or KTGQFL were included as covariates (independent of IgG or IgA reactivity). Using this model, the absence of measurable IgG or IgA against a SUMPP in an individual with measurable IgE against that SUMPP has a significant predictive effect in determining MS/control status ($p=0.0021$), while IgE positivity alone with respect to either HRTFE or KTGQFL is no longer significant ($p=0.1823$ and $p=0.2525$, respectively). Using a 2×2 contingency table and a chi-square test of independence, there is a significant ($p=0.0006$) association between being *overall positive* (IgE+/IgG-/IgA- against one or more SUMPP) and having MS. The estimated odds ratio is 14.62 with a 95% confidence interval of (2.656, 80.524). Together, these data suggest that IgE against certain CNS peptide antigens may be increased in MS patients and, moreover, that the lack of measurable IgG/IgA against a SUMPP demonstrating IgE reactivity may add diagnostic specificity.

4. Discussion

In theory, autoreactive Ig might: (1) be pathogenic, (2) be reparative (Asakura and Rodriguez, 1998; Pirko et al., 2004; Warrington et al., 2000), (3) represent an epiphenomenon occurring subsequent to CNS injury or (4) reflect nonspecific immune dysregulation (Cortese et al., 1996; Reder and Oger, 2004). In the case of complement-fixing antibodies or IgE, when target antigen is engaged, these antibodies are implicitly destructive, with mast cell degranulation occurring in the case of bivalent IgE binding to target. It has been theorized that, in the periphery, through their production of histamine and vasoactive amines, mast cells might disrupt the integrity of the blood–brain barrier and facilitate entry of autoreactive T_H1 cells into the CNS (Bebo et al., 1996). We theorize an additional mechanism whereby focal areas of intra CNS mast cell degranulation might occur as a consequence of intravascular myelin-reactive IgE penetrating the blood–brain barrier.

The finding of significantly more SUMPP-specific IgE in MS patients (Figs. 2 and 3) suggests that autoreactive IgE may contribute to MS pathogenesis. SUMPP-reactive IgE could be detected in controls, however. Thus, if IgE reactive against a component of the CNS is of relevance in MS, one must posit that either (1) reactive IgE detected in serum might not enter the CNS to the same degree in controls or (2) Ig of some other

isotype reactive with the same epitope might physically block IgE from binding to its target; or both of the above might hold true. It cannot be assumed that the relative levels of IgE, IgG and IgA within the CNS are reflected by serum levels, which is supported by findings of increased levels of anti-MOG IgG in MS brain compared to serum (O'Connor et al., 2005). We speculate that increased CNS-reactive IgE (or other harmful Ig) in CSF relative to serum (analogous to IgG index, but epitope-specific), in excess of potentially blocking Ig(s) in CSF, might be pathogenic. While the focus of this study is IgE, other Igs reactive with CNS antigens might be harmful via complement-dependent or independent pathways (Prineas and Graham, 1981). Considering the possibility that SUMPP-specific IgG or IgA might serve as blocking antibodies (Fig. 4), the sensitivity and specificity of this serum assay are 69% and 87%, respectively. Future work will investigate CSF/serum Ig reactivity against peptides.

A theoretical disadvantage of this assay, in its present methodology, is that it will not detect carbohydrate, lipid or non-linear protein epitopes; however, it is not yet certain whether non-proteins are a factor in MS pathogenesis. Moreover, if multiple reactive humoral determinants exist within an individual, it may be unnecessary to ascertain all reactive epitopes. Sensitivity and specificity might be improved by testing IgM (which could function as a “blocking” antibody, as theorized for IgG and IgA) by subclassifying IgG and IgA, and by identifying peptide epitopes that are truly specific to MS populations. Ongoing longitudinal studies, testing of a larger number of untreated MS patients, and testing of other types of controls (neurological, inflammatory, autoimmune, allergic) will help address the utility of this assay. It should be noted that the predictive power of this test might be improved by testing naive or currently untreated patients, as immunomodulatory or immunosuppressive treatments might alter the balance of Ig isotypes. Additional peptides from other myelin and neural proteins are being investigated, and non-CNS control peptides will be tested, along with measurement of total serum IgE, as one could otherwise postulate that our observations simply represent an IgE “shift” among individuals with inflammatory neurological disease. The fact that not all peptides were equally predictive argues against the latter possibility.

While MS has in general been characterized by a T_H1 response, it now appears that the destructive, inflammatory phase of MS is not strictly T_H1 -mediated. If MBP-specific T_H2 cells are passaged into immunodeficient mice, mice develop severe EAE that correlates with a large mast cell infiltrate in the meninges (Lafaille et al., 1997). Mast cells appear to play a role in other autoimmune diseases, such as rheumatoid arthritis, also considered to be a T_H1 disease (Benoist and Mathis, 2002; Lee et al., 2002). Pathogenic responses involving mast cells are dominated by T_H2 cells and are dependent upon the ability of antigen-specific IgE to bind to FcεRI expressed on mast cells. Cross-linkage of FcεRI results in mast cell activation and release of tumor necrosis factor α , various interleukins, as well as histamine, chemokines, prostaglandins, leukotrienes and

proteases (Krishnaswamy et al., 2006; Metcalfe et al., 1997). Together, these mediators have powerful effects on inflammatory cell activation and recruitment, chemokine production, endothelial adhesion molecule expression and vascular permeability. Mast cells have not been described in the CSF but are present in human brain (Brenner et al., 1994; Johnson and Krenger, 1992; Johnson et al., 1988; Lafaille et al., 1997; Silver et al., 1996) and their presence in the MS lesion has been known for several decades (Ibrahim et al., 1996; Kruger et al., 1990; Olsson, 1974; Prineas and Wright, 1978; Toms et al., 1990). IgE-positive cells, presumably plasma cells, have been identified in MS brains (Toms et al., 1990). Moreover, histamine (Tuomisto et al., 1983) and the mast cell marker tryptase (Rozniecki et al., 1995) are elevated in the CSF of MS patients, and mast cell proteases are capable of generating encephalitogenic myelin fragments *in vitro* (Dietsch and Hinrichs, 1991).

Steinman and colleagues have found that histamine type 1 receptor antagonists, a platelet activating factor receptor antagonist, and blockade of Ig Fc receptors (including FcεRI) reduce the severity of EAE (Dietsch and Hinrichs, 1989; Pedotti et al., 2003; Pedotti et al., 2001). These results support an important role for immune response elements associated with allergy in EAE. In addition, two groups have shown that mast cells are necessary for the full manifestation of MOG-induced EAE, and restoration of mast cells results in severe disease (Brown et al., 2002; Secor et al., 2000). It is of note that the histamine 1 receptor antagonist hydroxyzine has been shown to suppress intra-CNS mast cell degranulation and severity of neurological impairment in EAE (Dimitriadou et al., 2000), and an open label pilot study using this agent was promising (Logothetis et al., 2005).

The serum assay described herein is novel in that reactive IgE, IgG and IgA are quantified, short linear peptides are used rather than full-length myelin protein, and rather than considering all Igs equally, it classifies antibodies as being potentially harmful (IgE) or potentially protective, as we speculate that in the balance, Igs of different isotypes reactive with the same epitope might be associated with disease or not. To date, testing of myelin protein-specific serum IgG and IgM has not proved to be diagnostically useful when compared to controls, and therefore considering that some Igs may be protective or neutral is a plausible approach. This approach is supported by work showing that CSF IgA levels might be correlated with survival among MS patients (Vrethem et al., 2004).

In theory, CNS-specific autoantibody production is more likely to be targeted against peptide regions that share minimal structural overlap with peptide regions of other endogenous proteins. Such a region might become an autoimmune epitope in an individual whose immune responses, dictated by their major histocompatibility complex repertoire, allows them to break tolerance if stimulated by a homologous region on a foreign protein. This provides a theoretical basis for peptide selection and is supported by our previous unpublished work, as exemplified by Fig. 1. MS is

Table 2
Homology of SUMPP sequences to non-mammalian polypeptides

	Bacteria	Fungi	Insects	Plants	Viruses
<i>OSP</i>					
AHRET	11	8	1	2	2 (plant)
<i>MOG</i>					
AAMEL	4	2	3	0	0
HSYQE	1	0	1	0	0
QAPEY	6	3	0	0	0
VTLRI	15	0	1	0	0
HRTFE	4	0	0	0	0
KTGQFL	1	0	0	0	0
<i>PLP</i>					
ADARM	20	5	1	1	2 (human)

SUMPPs were chosen for analysis based on the lack of significant sequence similarity to other human proteins. Blast search of the eight SUMPPs reveals sequence identity with a number of infectious and environmental agents, as shown. It is theorized that, through molecular mimicry, exogenous agents could trigger an immune response and, if the target of attack is a SUMPP sequence, augmentation of a CNS-specific autoimmune process could result.

an autoimmune disease shaped by genetic susceptibility, but the environmental triggers remain unknown. It is generally believed that a pathogen may be causal, and that immunopathogenesis follows on the basis of molecular mimicry (Wekerle and Hohlfeld, 2003) with as yet unidentified neural antigens. Upon searching the tested peptide sequences across all species, sequence identity is seen among infectious and other environmental agents, such as plants (Table 2). It is tempting to speculate that such agents might play a role in initiation or persistence of an antibody response directed against epitopes shared with CNS target antigens.

In summary, there is increasing evidence that the humoral arm of the immune system plays an important role in MS. A challenge lies in that measurable CNS-reactive serum autoantibodies may or may not be harmful in and of themselves. Our work suggests that serum IgE against myelin protein-derived peptides may be increased in MS. Ongoing work indicates that reactive IgE is increased to a greater extent in MS patients with progressive disease as compared to RRMS. We speculate that autoreactive IgE may be pathogenic, and that non-IgE antibodies may be protective if present at much higher concentrations. We propose that, when measuring serum Ig reactive against CNS antigens, the spectrum of different Ig isotypes should be considered. Better delineation of autoreactive Igs may enable immunological phenotyping of MS patients, facilitate development of a diagnostic test and lead to new treatment directions.

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