Antibody Cross-Reactivity between Myelin Oligodendrocyte Glycoprotein and the Milk Protein Butyrophilin in Multiple Sclerosis[1,2]

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The etiology of multiple sclerosis (MS) is believed to involve environmental factors, but their identity and mode of action are unknown. In this study, we demonstrate that Ab specific for the extracellular Ig-like domain of myelin oligodendrocyte glycoprotein (MOG) cross-reacts with a homologous N-terminal domain of the bovine milk protein butyrophilin (BTN). Analysis of paired samples of MS sera and cerebrospinal fluid (CSF) identified a BTN-specific Ab response in the CNS that differed in its epitope specificity from that in the periphery. This effect was statistically significant for the Ab response to BTN_{76-100} ($p = 0.0026$), which cosequestered in the CSF compartment with Ab to the homologous MOG peptide MOG_{76-100} in 34% of MS patients ($n = 35$). These observations suggested that intratheccal synthesis of Ab recognizing BTN peptide epitopes in the CNS was sustained by molecular mimicry with MOG. Formal evidence of molecular mimicry between the two proteins was obtained by analyzing MOG-specific autoantibodies immunopurified from MS sera. The MOG-specific Ab repertoire cross-reacts with multiple BTN peptide epitopes including a MOG/BTN_{76-100}-specific component that occurred at a higher frequency in MS patients than in seropositive healthy controls, as well as responses to epitopes within MOG/BTN_{1-39} that occur at similar frequencies in both groups. The demonstration of molecular mimicry between MOG and BTN, along with sequestration of BTN-reactive Ab in CSF suggests that exposure to this common dietary Ag may influence the composition and function of the MOG-specific autoimmune repertoire during the course of MS. *The Journal of Immunology,* **2004, 172: 661–668.**

The etiology of multiple sclerosis (MS)[4] involves environmental factors that are believed to disrupt immunological self-tolerance to CNS myelin in genetically susceptible individuals (1, 2). However, the identity of these factors and how they might trigger or exacerbate autoimmune responses to specific myelin autoantigens is unknown. One mechanism discussed in this context is molecular mimicry (3); the induction of autoimmunity due to the presence of shared sequence or structural homologies with a foreign Ag (4). Many peptides derived from common viruses share linear sequence homologies with myelin proteins, and in animal models these can induce cross-reactive and potentially pathogenic T cell responses (5–10). This cross-reactive response reflects the degeneracy of peptide-MHC complex recognition by the TCR, which allows a single receptor to bind a hierarchy of peptide ligands (6, 11). However, while commonly discussed as a trigger for autoimmune disease, the pathophysiological significance of molecular mimicry during the natural course of an acute infection remains uncertain (12).

The route of sensitization will in part determine the outcome of molecular mimicry, a factor that becomes important if sensitization occurs across the gastrointestinal tract. This will normally lead to oral tolerance, a physiological response that suppresses potentially inflammatory T cell responses to Ag derived from the diet or the gut microbial flora (13). However, oral tolerance can be disrupted by concurrent gastrointestinal infections (14, 15) and is also poorly developed in suckling neonates (13, 16, 17), situations in which mimicry between dietary Ags and self could result in autoaggression. The potential importance of mimicry involving dietary Ags for MS was first recognized following the demonstration of immunological cross-reactivity between bovine milk proteins and CNS myelin autoantigens in an animal model of MS, experimental autoimmune encephalomyelitis (EAE) (18, 19). These cross-reactive immune responses involved epitopes derived from myelin basic protein and BSA (18), and the milk protein butryophilin (BTN) and myelin oligodendrocyte glycoprotein (MOG) (19).

MOG was identified as a candidate autoantigen in MS following the demonstration that MOG-induced EAE reproduced the immunopathology and complex clinical course of the human disease in rodents and primates (20–22). MOG is localized at the outer surface of the CNS myelin sheath where it can be targeted by demyelinating autoantibody responses directed against its extracellular N-terminal Ig-like domain (MOG^{Igd}) (23, 24). In MOG-induced

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[4] Abbreviations used in this paper: MS, multiple sclerosis; MOG, myelin oligodendrocyte glycoprotein; MOG^{Igd}, MOG Ig domain; EAE, experimental autoimmune encephalomyelitis; BTN, butyrophilin; BTN^{exo}, BTN exoplasmic domain; BTN^{Igl}, BTN N-terminal Ig domain; CSF, cerebrospinal fluid; HD, healthy control donor.

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EAE, this demyelinating Ab response acts synergistically with an encephalitogenic MOG-specific T cell response to reproduce the inflammatory demyelinating pathology of MS (21, 22). Reports of enhanced MOG-specific T cell (25–28) and Ab (28–31) responses in MS patients suggest that autoimmunity to MOG may play a similar role in the pathogenesis of human disease. This concept is supported by the demonstration of MOG-reactive Abs associated with disintegrating vesicular myelin debris in acute demyelinating MS lesions (22, 32) and a recent report that MOG-specific Ab can be used as a prognostic marker early in the course of disease (33).

In contrast to MOG, BTN is expressed only in the lactating mammary gland where it forms a major component of the milk fat globule membrane (34). The two proteins are members of an extended family of B7-like proteins encoded by single genes located telomeric to the HLA complex (35) that are related by the structure and amino acid sequence of their N-terminal Ig V-like domains, which have an amino acid sequence identity of ~50% (36). The ease with which self-tolerance to MOG is disrupted in both EAE and MS is attributed to the inability of the protein to induce self-tolerance. The expression of MOG protein outside the immunologically privileged environment of the CNS is controversial (37, 38). However, if MOG is expressed in immune organs, recent studies using genetically manipulated MOG-deficient mice demonstrate that MOG itself is unable to induce immunological self-tolerance (39). As a consequence, potentially pathogenic MOG-reactive lymphocytes are retained within the healthy immune repertoire and may be activated due to mimicry with epitopes derived from environmental agents (8, 19, 40). In the case of BTN, immunization in CFA activates a cross-reactive Th1 $CD4^+$ T cell response to MOG that initiates a subclinical encephalomyelitis (19). However, if sensitization is transmucosal, molecular mimicry between the two proteins can be exploited to induce a protective immune response that suppresses MOG^{Igd}-induced EAE (19).

Epidemiological studies repeatedly associate the prevalence of MS with dietary factors including the consumption of milk and dairy produce (41–43), and this has lead to speculation that molecular mimicry involving BTN may modulate MOG-specific autoimmune responses in humans (19, 44). To examine this possible link in more detail, we investigated the Ab response to MOG^{Igd} in patients with MS for evidence of molecular mimicry with BTN. We report that MOG^{Igd}-specific autoantibodies immunopurified from MS sera cross-react with multiple epitopes present within the N-terminal Ig domain of the protein (BTN^{IgI}). Furthermore Ab responses to certain BTN peptides are preferentially sequestered in the CNS, suggesting they may be involved in disease pathogenesis. These results provide the first formal demonstration of molecular mimicry involving this common dietary Ag in MS and suggest that the composition and function of the MOG-specific immune repertoire may be influenced during the course of disease by BTN present in milk and dairy products.

Materials and Methods

Ags

Recombinant human MOG^{Igd} (aa 1–125) corresponding to the N-terminal Ig-like domain of the protein and rat S100β were expressed with a C-terminal hexahistidine tag in *Escherichia coli* and purified as described previously (31). The entire mature exoplasmic domain of bovine BTN (aa 1–216; BTN^{exo}; Ref. 34) was expressed as a baculo virus product in High5 cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA) and purified by chromatography on Ni-NTA agarose to a purity of >98% as assessed by gel electrophoresis. Protein concentration was determined using Peterson's modification of the micro-Lowry method (Sigma-Aldrich, Deisenhofen, Germany). Panels of overlapping synthetic peptides spanning both MOG^{Igd} and BTN^{IgI} were purchased from Genosys (Cambridge, U.K.) (Table I).

Table I. *Synthetic human MOG and bovine BTN peptides used in this study*[a]

	Amino Acid Sequence
P1	
MOG_{1-26}	GQFRVIGPRHPIRALVGDEVELPCRI
BTN_{1-26}	APFDVIGPQEPILAVVGEDAELPCRL
P2	
MOG_{14-39}	ALVGDEVELPCRISPGKNATGMELGW
BTN_{14-39}	AVVGEDAELPCRLSPNVSAKGMELRW
P3	
MOG_{27-50}	SPGKNATGMELGWYRPPFSRVVHL
BTN_{27-50}	SPNVSAKGMELRWFREKVSPAVFV
P4	
MOG_{38-60}	GWYRPPFSRVVHLYRNGKDQDGD
BTN_{38-60}	RWFREKVSPAVFVSREGQEQEGE
P5	
MOG_{50-74}	LYRNGKDQDGDAPEYRGRTELLKD
BTN_{50-74}	VSREGQEQEGEMAEYRGRVSLVED
P6	
MOG_{63-87}	PEYRGRTELLKDAIGEGKVTLRIRN
BTN_{63-87}	AEYRGRVSLVEDHIAEGSVAVRIQE
P7	
MOG_{76-100}	IGEGKVTLRIRNVRFSDEGGFTCFF
BTN_{76-100}	IAEGSVAVRIQEVKASDDGEYRCFF
P8	
MOG_{89-113}	RFSDEGGFTCFFRDHSYQEEAAMEL
BTN_{89-113}	KASDDGEYRCFFRQDENYEEAIVHL
P9	
$MOG_{101-125}$	RDHSYQEEAAMELKVEDPFYWVSPG
$BTN_{101-120}$	RQDENYEEAIVHLKVAALGS

[a] Amino acid sequences of overlapping synthetic peptides spanning the N-terminal domains of human MOG (MOG^{Igd}; accession number I56513) and bovine BTN (BTN^{IgI}; accession number M35551). Amino acid residues conserved between the two proteins are underlined.

Patients, control donors, and Ab purification

Blood and cerebrospinal fluid (CSF) sampling techniques were approved by the Karolinska Institute Ethical Committee and samples were taken after obtaining the donors informed consent. A total of 35 paired samples of serum and CSF was obtained from patients with clinically definite MS as defined using the Poser criteria and who were undergoing no immunotherapy at the time (males, $n = 13$; mean age, 43.8 years; range, 25–60 years; females, $n = 22$; mean age, 40.6 years; range, 24–59 years). Control sera were obtained from a group of 25 healthy donors (males, $n = 9$; mean age, 35.3 years; range, 28–41 years; females, $n = 16$; mean age, 35 years; range, 24–56 years).

MOG^{Igd}-reactive Abs were isolated from peripheral blood (200–300 ml) taken with informed consent from 12 MS patients (males, $n = 4$; mean age, 40 years; range, 17- 54 years; females, $n = 8$; mean age, 46 years; range, 21–58 years) and 9 MOG Ab-seropositive healthy donors (males, $n = 6$; mean age, 35.5 years; range, 29–46 years; females, $n = 3$; mean age, 27 years; range, 24–30 years). In addition, samples were also obtained from a patient with an acute steroid-unresponsive, intractable relapse who required plasmapheresis (female, age 28) and from one patient with a chronic progressive course who regularly undergoes lipid (low-density lipoprotein) pheresis for hyperlipidemia (female, age 43).

MOG^{Igd}-specific autoantibodies were isolated as described previously (31). Briefly, serum and plasma samples (~150 ml) were diluted 1/1 with PBS and passed through a matrix consisting of human MOG^{Igd} coupled to cyanogen bromide-activated agarose (Sigma-Aldrich) at 4°C. The matrix was then washed extensively with PBS until the absorbance of the wash buffer flow-through had returned to its baseline A_{280} value. Bound Abs were eluted with 0.1 M glycine (pH 2.5) immediately neutralized by the addition of concentrated (10×) PBS (pH 7.4) and analyzed without any further manipulations.

ELISA

ELISA was performed using polystyrene 96-well PVC plates (Costar, Cambridge, MA) coated overnight with 10 μg/ml Ag in PBS containing 0.02% NaN_3. The plates were then washed with PBS containing 0.05% Tween 20/0.02% NaN_3 and blocked with 1% (w/v) BSA in PBS for a minimum of 1 h at 37°C. The plates were again washed with PBS-Tween 20 and incubated with 100 μl of diluted serum/Ab either overnight at 4°C

or alternatively for 1 h at 37°C. Bound Ab was detected using 100 μl of either peroxidase or alkaline phosphatase-conjugated, human IgG-specific Abs diluted in PBS (Dianova, Hamburg, Germany). Plates were developed with either *o*-phenyldiamine or *p*-nitrophenyl phosphate (Sigma-Aldrich) as appropriate and OD was determined either at 490 or 405 nm, respectively. The background OD varied between samples and the Ag-specific response was only considered positive when it exceeded a threshold defined as the background plus 2 SDs in wells coated with BSA and incubated with both sample and secondary Ab. Statistical evaluation was performed using the Student's *t* test, the Fisher's exact test, or the McNemar test, as indicated in the text.

Results

Serum Ab responses to BTN^{IgI} epitopes in MS patients

Previous studies reported that MS is associated with increased levels of serum Ab to several milk proteins (18), including BTN (44), but in this study we found that this was not the case for the Ab response to epitopes within BTN^{IgI}, the N-terminal region of BTN (aa 1–120) homologous to MOG^{Igd}. Analysis of the serum Ab response to the entire exoplasmic domain of BTN (aa 1–216; BTN^{exo}) revealed no significant differences between MS patients and healthy control donors (HD)(Fig. 1). In both groups, the frequency of responses to BTN^{exo} was >85% (MS, $n = 35$, frequency = 89%; HD, $n = 25$, frequency = 88%), and there was no significant difference in the mean Ab response (MS, mean $A_{405} = 0.43$; HD, mean $A_{405} = 0.54$; *t* test, $p > 0.05$). However, the frequency of responses to individual BTN^{IgI} peptides was consistently lower in MS patients than HD (Fig. 1*b*). One or more peptides were recognized by 75% of HD, but by only 54% of the MS patients (Fig. 1*b*). This disease-associated effect was observed for all BTN^{IgI} peptides but appears more pronounced for responses directed toward epitopes within the C-terminal half of this domain. This decrease in Ab responses to individual BTN^{IgI} peptides is apparently masked by increased reactivity to other regions of the protein when the BTN-specific Ab response is assayed using either BTN^{exo} (see above) or the full-length protein (44).

Ab responses to individual BTN^{IgI} peptides in MS patients are differentially distributed in sera and CSF

To investigate whether the decreased serum Ab response to BTN^{IgI} peptides in MS was due to sequestration within the CNS, we analyzed the patients' CSF. Comparison of Ab responses in paired sera and CSF samples ($n = 35$) identified a subset of 15 patients with a CSF Ab response to one or more BTN^{IgI} peptides (Table II). In many cases, this CSF response was not accompanied by a corresponding peptide-specific serum Ab response (Table II), resulting in a strikingly different specificity profile in the serum and CSF (Fig. 2*a*). In the sera the response to BTN^{IgI} epitopes was dominated by Ab recognizing BTN$_{1–26}$. This specificity was present in 43% of sera (15 of 35), but in only 9% of CSF samples (3 of 35; $p = 0.0033$, McNemer test). In contrast, the frequency of Ab responses to all other BTN^{IgI} peptides was higher in CSF, where the response was dominated by Ab recognizing peptide BTN$_{76–100}$ (Fig. 2*a*). This specificity was detected at a high level in 34% of CSF (12 of 35; A_{405}, range, 0.20–2.16; mean, 0.54), but in only one serum sample ($p = 0.0026$, McNemer test). Concordance of CSF Ab responses to the BTN^{IgI} peptide(s) and recombinant protein was 66% and in six cases the response to recombinant BTN was higher in the CSF than in sera suggestive of local intratheccal Ab synthesis (Table II). These observations reveal that Ab responses to certain BTN^{IgI} peptides are differentially distributed between the periphery and CNS, in particular Abs binding to BTN$_{76–100}$ are selectively sequestered in the CNS, while Ab responses to BTN$_{1–26}$ are skewed in favor of serum

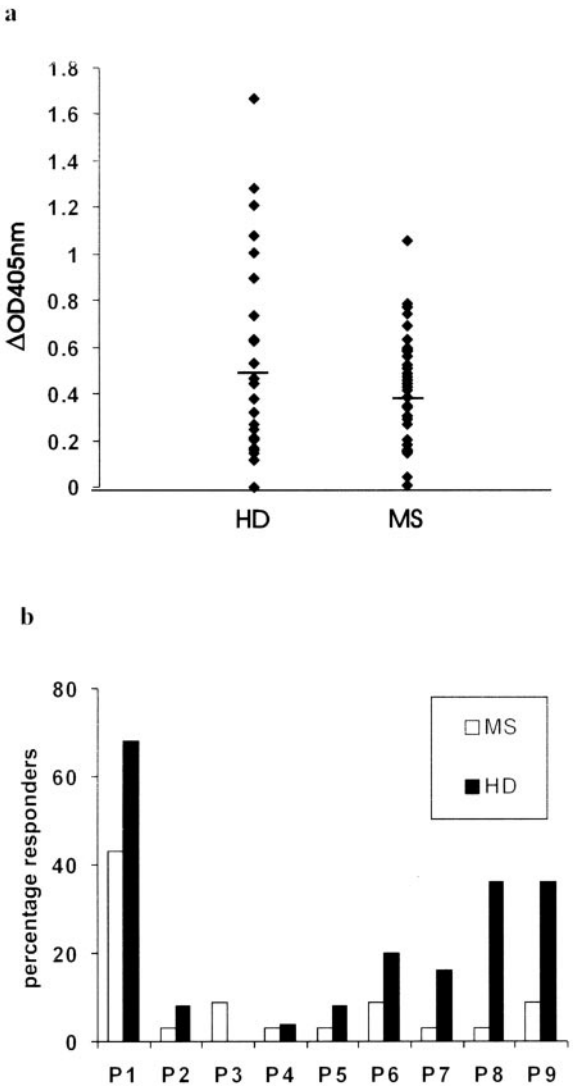

FIGURE 1. Serum Ab responses to BTN^{exo} and BTN^{IgI} peptides. *a*, Serum Ab responses to the exoplasmic domain of BTN were determined in the sera of healthy controls ($n = 25$) and MS patients ($n = 35$) by ELISA. The frequency of seropositive responders was 88% in the control population (22 of 25) and 89% in the cohort of MS patients (31 of 35). The mean OD values for the two groups are indicated by the horizontal bars and were not significantly different (MS, 0.43; HD, 0.54; $p > 0.05$, Student's *t* test). Each value represents the mean OD corrected for background for a single donor assayed in quadruplicate. *b*, The frequency of Ab responses to BTN^{IgI} was determined by ELISA in MS patients ($n = 35$) and healthy controls ($n = 25$) using overlapping synthetic BTN^{IgI} peptides (Table I). Donors were regarded as seropositive when the mean OD obtained against a peptide exceeded the background value by at least 2 SDs. All assays were performed in quadruplicate.

Abs to BTN$_{76–100}$ cosegregate with the response to MOG$_{76–100}$ in the CNS

The sequestration of Ab responses recognizing a bovine milk protein in the CNS was surprising and led us to speculate that this may reflect intratheccal Ab synthesis stimulated by molecular mimicry with the homologous region of MOG^{Igd}. The patients' Q Albumin (Q_{Alb}) values support the concept that Ab synthesis is occurring within the CNS compartment (Table II), but it was not possible to test for molecular mimicry directly, since insufficient CSF was available to either immunopurify BTN$_{76–100}$ Ab for direct analysis

Table II. *BTN^{IgI} peptide-specific Abs are sequestered in CSF*[a]

Donor	Q_{Alb}		ΔOD_{490}									
			P1	P2	P3	P4	P5	P6	P7	P8	P9	BTN
10	5.3	CSF	—[b]	—	—	—	—	—	0.39	—	0.16	0.83
		Serum	—	—	—	—	—	—	—	—	—	0.18
12	5.7	CSF	—	—	—	—	—	—	0.20	—	—	1.24
		Serum	—	—	0.21	—	—	—	—	—	—	0.15
17	6.1	CSF	0.32	0.17	—	—	—	0.22	—	—	—	0.67
		Serum	—	—	—	—	—	—	—	—	—	0.47
19	4.4	CSF	—	—	—	—	0.25	0.15	0.32	0.26	—	0.77
		Serum	0.35	—	—	—	—	—	—	—	—	0.16
20	8.0	CSF	—	—	0.27	0.24	—	—	0.20	0.32	0.29	0.29
		Serum	—	—	—	—	—	—	—	—	—	—
22	4.9	CSF	—	0.16	0.10	0.12	0.23	0.29	0.25	0.12	0.33	0.30
		Serum	—	—	—	—	—	—	—	—	—	—
23	6.5	CSF	0.47	—	—	—	—	—	0.74	—	—	0.43
		Serum	0.14	—	—	—	—	—	—	—	—	0.29
33	5.7	CSF	—	0.19	0.10	0.17	0.13	0.05	0.26	0.05	0.17	—
		Serum	0.21	—	—	—	—	—	—	—	—	0.39
34	7.4	CSF	—	—	0.09	—	—	0.10	0.28	—	—	0.10
		Serum	—	—	—	—	—	—	—	—	—	0.15
37	4.5	CSF	—	—	—	—	—	—	2.16	—	—	0.12
		Serum	—	—	—	—	—	—	0.89	—	—	0.42
40	2.9	CSF	—	—	0.13	—	—	—	0.55	—	—	—
		Serum	—	—	—	—	—	—	—	—	—	0.27
42	4.3	CSF	—	0.08	0.18	0.08	—	0.09	0.20	0.15	—	—
		Serum	0.60	—	—	—	—	—	—	—	—	0.34
48	12.3	CSF	—	0.16	0.11	0.36	—	0.21	0.94	0.28	0.10	0.22
		Serum	0.16	—	—	—	—	—	—	—	—	0.20
75	4.9	CSF	—	—	—	—	—	—	—	—	0.20	—
		Serum	0.08	—	—	—	—	—	—	—	—	0.56
97	4.3	CSF	0.11	—	—	—	—	—	—	—	—	—
		Serum	0.57	—	—	—	—	0.09	—	—	—	0.69

[a] A subset of 15 patients ($n = 35$) were identified with Ab responses to one or more BTN peptides in their CSF. With the exception of the response to peptide P1 ($BTN_{1–26}$), the majority of BTN peptide-specific responses in this subset of patients were restricted to the CNS compartment. Sera (diluted 1/30) and CSF (diluted 1/5) were analyzed in quadruplicate; samples were regarded as positive when the mean OD exceeded the background value by at least 2 SDs. Data are presented as the mean OD corrected for background.

[b] —, No significant response.

or establish competition assays with MOG and MOG-derived peptides. However, comparison of the specificity profiles of the BTN^{IgI} and MOG^{Igd} peptide-specific response in sera and CSF provides circumstantial evidence that this may indeed be the case. Anti-MOG^{Igd} Ab responses were detected at similar high frequencies in both MS sera (72%) and CSF (77%). The epitope specificity of this Ab response was heterogeneous and did not exhibit such an obvious pattern of sequestration within the CNS as observed for BTN (Fig. 2*b*). Nevertheless, we still observed a selective and statistically significant skewing of the response to $MOG_{76–100}$ in favor of CSF that mimics the response to the homologous BTN peptide. Abs binding to $MOG_{76–100}$ were present in 60% of CSF but in only 9% of sera ($p = 0.001$, McNemer test). A similar skewing of the Ab response in favor of the CSF was also seen for the peptides $MOG_{89–113}$ and $MOG_{101–125}$, but in this case the differences were not statistically significant. All patients with a CSF Ab response to $BTN_{76–100}$ had a corresponding response to $MOG_{76–100}$ and regression analysis of the data obtained from all 35 patients supports the proposal that these responses selectively cosegregate in the CNS compartment (Fig. 2*c*; $n = 35$, $R^2 = 0.681$).

MOG^{Igd}-specific autoantibodies cross-react with BTN peptide epitopes

To confirm that molecular mimicry can occur between the two proteins, in particular within the amino acid sequence 76–100, we investigated the ability of immunopurified MOG^{Igd}-specific Igs to bind to BTN^{IgI} peptides. MOG^{Igd}-specific Igs were isolated from sera/plasma of 14 MS patients and 9 seropositive coworkers (31). The specificity of the Igs eluted from the MOG^{Igd} matrix was confirmed by ELISA and Western blotting, the latter also being used to control for potential contamination by anti-bacterial Abs (data not presented).

Epitope mapping revealed that immunopurified MOG^{Igd}-specific Abs could bind several BTN^{IgI} peptides (Table III). This cross-reactive response was heterogeneous, but dominated by two distinct clusters of epitopes defined by the overlapping peptides $BTN_{1–26}$ and $BTN_{14–39}$ and $BTN_{50–74}$ and $BTN_{63–87}$. Cross-reactive Ab responses involving these two regions of BTN were present at similar frequencies in MS patients and HD: $BTN_{1–39}$, 64% of MS (9 of 14) and 78% of the controls (7 of 9); $BTN_{54–87}$, 50% of donors in both groups (Table III). In contrast, Ab cross-reacting with $BTN_{76–100}$ was detected more often in MS patients (43%, 6 of 14) than in control donors (11%, 1 of 9; Table III). This difference does not however reach statistical significance (Fisher's exact test, $p > 0.05$). This cross-reactive response between the MOG-specific Ab repertoire and BTN is biased in favor of cryptic peptide epitopes, as demonstrated when the assay was repeated using recombinant BTN as the target Ag), which revealed that only 6 of the 21 samples analyzed exhibited a cross-reactive response to recombinant BTN (Table IV).

MOG-reactive B cells are not thought to be deleted from the immune repertoire (39) and we anticipated that components of this "naive" MOG-reactive Ab repertoire that cross-react with BTN might be selectively expanded due to the presence of BTN in the diet. This was investigated using MOG^{Igd} binding Ab isolated

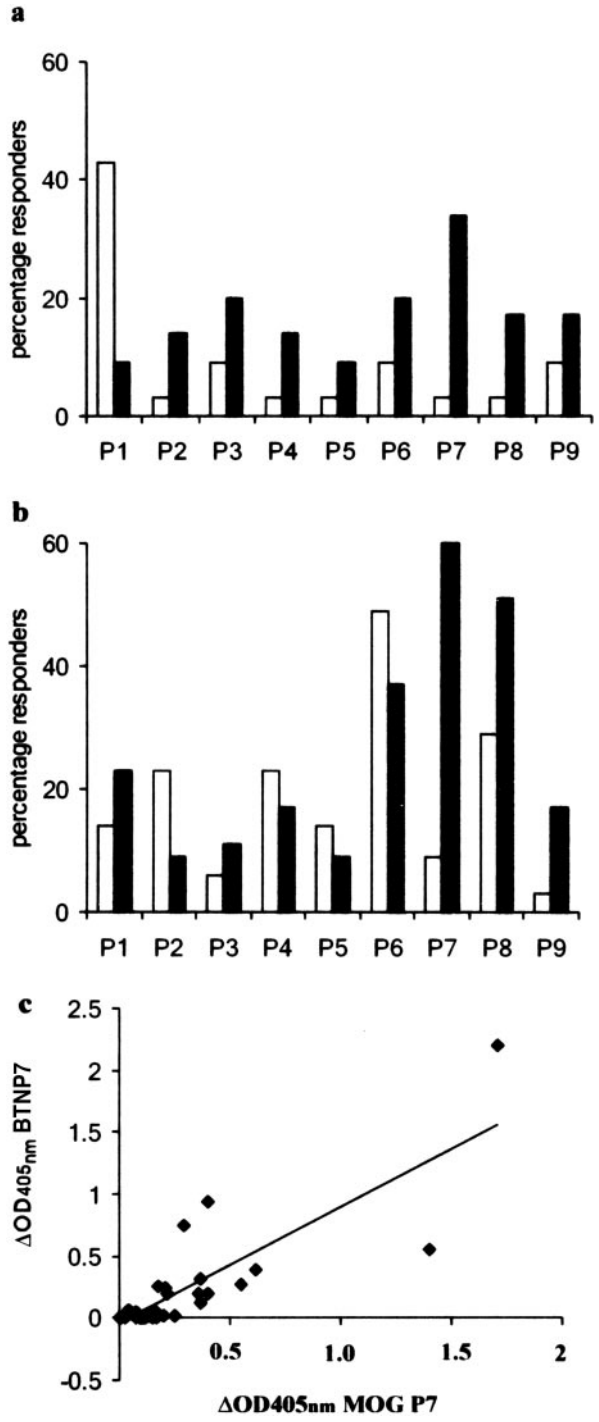

FIGURE 2. Differential patterns of epitope recognition in MS sera and CSF. The Ab response to BTN^{IgI} (*a*) and MOG^{Igd} (*b*) peptides was assayed in paired samples of MS sera (□) and CSF (■; $n = 35$). The bars represent the percentage of donors responding to each peptide. Note the high frequency of CSF responses to peptides spanning amino acid sequences 76–100 of both BTN^{IgI} and MOG^{Igd}. *c*, Comparison of Ab responses to MOG_{76-100} and BTN_{76-100} in CSF for all 35 MS patients ($R^2 = 0.681$). Data are presented as the mean OD corrected for background obtained from samples assayed in quadruplicate.

from a commercial Ig preparation (Sandoglobin: Novartis, Nürnburg, Germany) prepared from a collection of several thousand healthy donors. This approach allowed us to concentrate and isolate a small sample of this minor component of the naive repertoire. Surprisingly, there was no significant cross-reactivity between this Ab preparation and BTN^{IgI} peptides (Fig. 3). This was not due to the absence of Ab to regions of MOG^{Igd} associated with the cross-reactive response with BTN epitopes in seropositive donors, as Ab recognizing MOG_{1-26} and MOG_{14-39} was the major component of this naive repertoire (Fig. 3).

Discussion

In this study, we demonstrate that the autoimmune response to MOG, an important candidate autoantigen in MS, cross-reacts with the milk protein BTN. Modulation of the autoimmune repertoire by environmental factors is implicated in the etiology of several tissue-specific diseases with an underlying autoimmune pathogenesis (2, 45), but their identity and mode of action remain obscure. In MS, immune cross-reactivity to self-Ags triggered by microbial peptides is considered as one mechanism that may disrupt self-tolerance to CNS myelin Ags and initiate autoaggression (2). The extension of this concept to a common dietary Ag introduces new perspectives that are largely irrelevant in the context of acute microbial infections, in particular as the route of sensitization may induce a protective rather than an autoaggressive cross-reactive T cell response.

It has been suggested that increased Ab reactivity to dietary Ags in MS may reflect a generalized regulatory defect in mucosal immunity (18); a hypothesis supported by the concordance of inflammatory bowel disorders with MS (46) and the identification of susceptibility loci common to both MS and inflammatory autoimmune diseases affecting the gastrointestinal tract (47). Previous studies describe a disease-associated increase in the serum Ab response to several milk proteins, including BTN (18, 44). However, our data indicate that this is not the case for the Ab response to individual BTN^{IgI} peptides that occur at a lower frequency in MS than in healthy controls. This may be due to the sequestration or absorption of Ab with these specificities in the CNS and we surmise that any decrease in the serum Ab response to BTN^{IgI} peptides was masked in previous studies by enhanced responses to other regions of the protein (44). This would also account for the similar level of Ab reactivity to the entire exoplasmic domain of BTN in MS and control donors seen in this study.

Our evidence for molecular mimicry between MOG and BTN is currently restricted to the humoral arm of the immune system and is based on the analysis of immunopurified MOG^{Igd}-specific Ab isolated from sera obtained from both MS patients and healthy seropositive controls. The epitope specificity of the response is complex and also involves cryptic BTN peptide epitopes. Surprisingly, we were unable to detect an equivalent response in Ab isolated from a large pool of healthy naive donors. This observation along with the high frequency of BTN^{IgI}-specific Ab responses in the general population suggest that the expansion and selection of cross-reactive B cell clones is not driven by exposure to BTN per se, but rather the homologous MOG peptide epitope. In the majority of the population, MOG will remain sequestered behind the blood-brain barrier, but becomes accessible to the immune system in MS as a consequence of CNS inflammation, demyelination, and blood-brain barrier dysfunction, while the inhalation of MOG-containing aerosols apparently has a similar effect in laboratory workers (28). We are currently investigating whether cross-reactive MOG/BTN Ab responses are also enhanced in other CNS diseases in which loss of tolerance to MOG is reported to occur.

The pathophysiological consequences of Ab cross-reactivity between MOG and BTN are uncertain and its effects may well be epitope specific, as suggested by the differential distribution of responses to BTN_{1-26} and BTN_{76-100} between the periphery and CNS. The peptide specificity of the cross-reactive Ab response is probably influenced by the donors HLA haplotype, although we

Table III. *MOG^{Igd}-specific autoantibodies recognize BTN peptides*[a]

Donor Identifier	Sex	Age (years)	Disease Course	MOG	P1	P2	P3	P4	P5	P6	P7	P8	P9
MS Patients													
GT	F	28	P	1.95	—[b]	0.26 (0.79)	—	—	—	0.47 (0.46)	—	—	—
MT	F	43	P	2.35	—	—	0.52 (0.52)	—	—	—	0.51 (2.33)	—	2.41 (0.26)
19	F	53	SCP	2.62	—	—	—	—	—	—	—	—	—
20	F	28	RR	2.66	0.14 (0.50)	—	—	—	1.03 (0.17)	0.14 (1.76)	1.01 (0.12)	—	—
23	F	58	RR	2.88	—	—	—	—	0.42 (0.09)	—	—	—	—
24	F	50	RR	2.43	0.32 (0.83)	0.76 (0.21)	0.27 (1.82)	—	—	—	0.27 (0.13)	—	—
25	F	53	SCP	1.95	—		—	—	—	—	0.28 (0.14)	—	—
27	F	57	RR	2.67	—	0.38 (0.13)	0.19 (0.47)	—	0.17 (0.90)	0.08 (1.50)	0.93 (1.55)	0.21 (0.44)	—
28	M	45	RR	2.86	—	0.17 (0.42)	—	—	0.41 (0.04)	—	—	—	—
29	M	54	RR	2.31	0.25 (0.84)	—	—	—	0.20 (1.13)	—	—	—	—
30	M	44	SCP	2.50	0.10 (0.30)	0.11 (0.29)	—	—	—	0.05 (0.96)	0.06 (0.29)	—	—
31	M	17	RR	2.69	0.13 (0.14)	—	—	—	—	—	—	—	—
32	F	48	SCP	2.83	0.16 (0.26)	—	—	—	—	—	—	—	—
33	F	21	RR	2.73	—	—	—	—	—	0.13 (1.39)	—	—	—
Controls													
CL[c]	M	46	HD	1.29	0.48 (0.21)	0.30 (1.38)	—	0.14 (0.16)	0.62 (0.47)	0.19 (0.11)	—	—	—
Ast[c]	M	29	HD	1.01	—	0.86 (2.28)	—	—	1.81 (0.65)	0.61 (0.06)	—	—	—
01	F	27	HD	2.10	0.10 (0.38)	—	—	—	—	—	—	—	—
02	F	24	HD	0.97	0.12 (0.18)	—	—	—	0.08 (0.15)	—	—	—	—
03	M	30	HD	1.34	—	0.23 (0.14)	—	—	—	—	—	—	—
04	F	30	HD	0.86	—	—	—	—	—	—	—	—	—
05	M	33	HD	2.32	—	0.39 (0.24)	—	—	0.12 (0.28)	—	—	—	—
06	M	36	HD	1.75	—	0.82 (0.30)	—	—	—	—	0.39 (0.81)	0.21 (1.07)	—
07	M	39	HD	2.86	—	—	—	—	—	—	—	—	—

[a] MOG^{Igd}-specific autoantibodies were isolated from the sera/plasma of 14 MS patients and nine seropositive healthy controls by immunoaffinity chromatography and analyzed by ELISA as described in *Materials and Methods*. P, Primary progressive; RR, relapsing-remitting; SCP, secondary chronic progressive. The response to the homologous MOG peptide is given in parentheses below the OD obtained for the BTN peptides (29).

[b] —, No response. Similar patterns of reactivity were obtained when selected samples were reassayed. Data are presented as the mean OD_{490} corrected for background from assays performed in quadruplicate.

[c] These two donors were also exposed to BTN in the laboratory.

could not identify any specific association between haplotype and specificity in our limited sample set. In view of data suggesting that the immunodominant demyelinating Ab response to MOG is conformation dependent (31, 48–50), cross-reactive BTN/MOG Ab recognizing linear peptide epitopes may not mediate primary demyelination. Nevertheless, several residues within the N-terminal region of MOG^{Igd} (aa 1–2, 30 and 33–34) contribute to the binding of the demyelinating mAb 8-18C5 to MOG (50) and are therefore accessible to Ab in vivo. This raises the possibility that cross-reactive components of the Ab repertoire directed against the N-terminal sequence of MOG are selectively absorbed in the CNS by binding to MOG, a mechanism that may explain the low frequency of $BTN_{1–26}$-reactive Ab in the CSF compartment in MS. Similarly, some demyelinating MOG-specific mouse mAbs bind to peptides containing aa 63–87 (48), suggesting that BTN/MOG cross-reactive Ab recognizing this region of the molecule may also be pathogenic.

In contrast, the enhanced CSF Ab response to other BTN peptides, in particular $BTN_{76–100}$, indicates that these specificities are not rapidly cleared from the CNS, presumably because the cross-reactive epitopes are not accessible on the surface of the intact extracellular domain of MOG. The sequestration within the CNS of Ab specific for determinants of a milk protein is in itself surprising and we suggest that this may reflect intratheccal Ab synthesis maintained by molecular mimicry with the corresponding MOG epitope(s). This hypothesis is supported for Ab response to $BTN_{76–100}$ by the absence of detectable Ab in the majority of sera, the patients Q_{alb}, and the observation that Abs binding to $BTN_{76–100}$ and $MOG_{76–100}$ cosequester in the CNS. Moreover, molecular mimicry involving this region of the two proteins appears to be enhanced in the

Table IV. *Cross-reactivity between MOG^{Igd}-specific Abs and recombinant BTN*[a]

Donor	OD MOG	OD BTN	OD S100β	Donor	OD MOG	OD BTN	OD S100β
MS							
GT	2.15	0.13	0	32	2.21	0.14	0.05
MT	0.61	0.26	0.07	33	1.83	0.02	0.05
19	1.11	0.05	0.04	Controls			
20	1.06	0.10	0.10	CL	0.41	0	0
23	1.17	0.08	0.05	1	0.83	0.02	0.02
24	0.92	0.11	0.05	2	1.06	0.02	0
25	0.44	0.06	0.13	3	0.63	0.14	0.04
27	1.81	0.10	0.07	4	0.72	0.03	0.03
28	2.22	0.04	0.07	5	1.40	0.09	0.11
30	0.85	0.16	0.02	6	1.20	0.05	0.01
31	1.89	0	0	7	1.20	0.13	0.04

[a] Immunopurified MOG^{Igd}-specific autoantibodies isolated from the majority of donors were analyzed to investigate cross-reactivity with recombinant BTN. Assays were performed in quadruplicate and the data are presented as the mean OD corrected for the background obtained using BSA. Recombinant S100β was used as a control Ag to determine whether the immunopurified Abs had any significant reactivity with the recombinant His tag.

peripheral MOG^{Igd}-specific Ab repertoire of MS patients. A full understanding of the pathophysiological significance of Ab cross-reactivity involving these different regions of MOG/BTN will require a detailed mapping of the responses in serum and CSF combined with in vitro studies to investigate their effects on myelination and presentation of Ag to T cells.

The demonstration of molecular mimicry between these two proteins has broader implications with respect to MS that extends to the T cell repertoire. The consumption of milk and milk products provides a source of BTN-derived peptides that can cross the gut mucosa to stimulate Ag-specific immune responses both locally in gut-associated lymphoid tissue as well as in peripheral immune organs (51). This is a normal physiological event that induces oral tolerance, the systemic suppression of proinflammatory T cell responses to soluble dietary Ags (13). Mimicry involving BTN may therefore not only induce a cross-reactive B cell response but also a T cell response that is counterinflammatory and that may suppress cross-reactive and potentially encephalitogenic MOG-specific Th1 T cell responses, as indicated by the suppression of MOG-EAE by transmucosal treatment with BTN peptide (19). It should however be noted that oral tolerance can be abrogated by gastrointestinal infections (14, 15) that may allow a transient expansion of cross-reactive and encephalitogenic Th1 T cell responses to MOG that might exacerbate CNS inflammation. Similarly, oral tolerance is also poorly developed in suckling neonates (13, 16, 17) so that in the context of a susceptible genotype early exposure to bovine BTN could prime the MOG-reactive repertoire to potentiate disease activity later in life.

In summary, we demonstrate that the milk protein BTN acts as a molecular mimic of MOG and that immunological cross-reactivity occurs between these two proteins in a subset of MS patients. Because milk and milk products are a staple component of the Western diet, BTN should be considered a ubiquitous environmental factor that can influence the autoimmune response to this specific myelin autoantigen. The pathophysiological consequences of molecular mimicry involving BTN are difficult to predict, as they will be influenced by multiple factors, including an individual's genotype, the timing and level of exposure to BTN, and the health of the gastrointestinal tract. In fact, chance may play a major role in determining whether or not molecular mimicry between MOG and BTN leads to a detrimental or protective immune response in any particular individual. Intriguingly, epidemiological studies associate the prevalence of MS with the consumption of milk and dairy produce (41, 42, 43), but whether this is related to molecular mimicry involving MOG and BTN remains a matter of speculation.

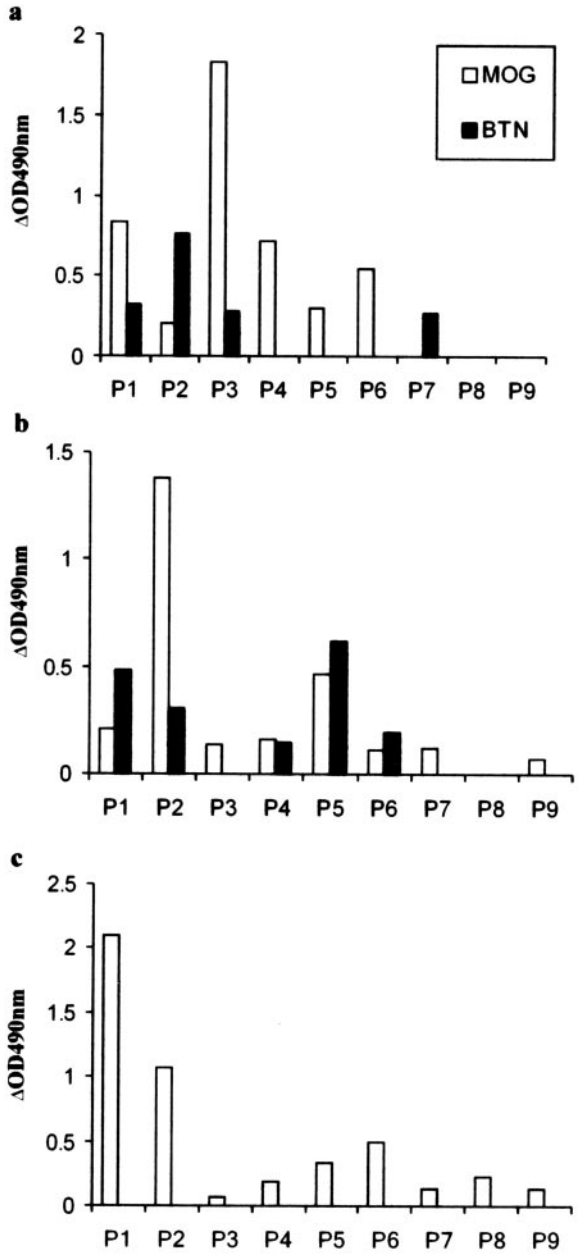

FIGURE 3. Recognition of BTN peptide sequences by immunopurified MOG^{Igd}-specific autoantibodies. Representative patterns of MOG (□) and BTN (■) peptide recognition by MOG^{Igd}-specific autoantibodies immunopurified from: *a*, a patient with MS (patient 24); *b*, a healthy donor exposed to MOG^{Igd} in the laboratory (control; CL); and *c*, pooled healthy, naive donors (Sandglobulin). Note that cross-reactivity with BTN peptides is absent in MOG^{Igd}-reactive Abs isolated from the commercial Ig preparation. OD values were determined in duplicate and corrected for nonspecific binding to BSA-coated plates. The homologous peptide sequences are defined in Table I.

Acknowledgments

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