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T Cells of Multiple Sclerosis Patients Target a Common Environmental Peptide that Causes Encephalitis in Mice¹

Shawn Winer,^{2*} Igor Astsaturov,^{2*} Roy K. Cheung,^{2*} Katrin Schrade,^{*} Lakshman Gunaratnam,^{*} Denise D. Wood,^{*} Mario A. Moscarello,^{*||} Paul O'Connor,[†] Colin McKerlie,[‡] Dorothy J. Becker,^{||} and Hans-Michael Dosch^{3*§}

Multiple sclerosis (MS) is a chronic autoimmune disease triggered by unknown environmental factors in genetically susceptible hosts. MS risk was linked to high rates of cow milk protein (CMP) consumption, reminiscent of a similar association in autoimmune diabetes. A recent rodent study showed that immune responses to the CMP, butyrophilin, can lead to encephalitis through antigenic mimicry with myelin oligodendrocyte glycoprotein. In this study, we show abnormal T cell immunity to several other CMPs in MS patients comparable to that in diabetics. Limited epitope mapping with the milk protein BSA identified one specific epitope, BSA₁₉₃, which was targeted by most MS but not diabetes patients. BSA₁₉₃ was encephalitogenic in SJL/J mice subjected to a standard protocol for the induction of experimental autoimmune encephalitis. These data extend the possible, immunological basis for the association of MS risk, CMP, and CNS autoimmunity. To pinpoint the same peptide, BSA₁₉₃, in encephalitis-prone humans and rodents may imply a common endogenous ligand, targeted through antigenic mimicry. *The Journal of Immunology*, 2001, 166: 4751–4756.

Multiple sclerosis (MS)⁴ is a chronic autoimmune disease of genetically susceptible hosts (1). Autoreactive T cells target constituents of myelin and oligodendrocytes for destruction, once a breach of the blood-brain barrier allows invasion by monocytes, dendritic cells, and effector T lymphocytes (2).

MS has much in common with autoimmune type 1 diabetes mellitus (T1DM), including near identical ethnic and geographic distribution and multiple genetic risk loci which overlap between the two diseases (3, 4). Much of the genetic susceptibility to MS and diabetes was mapped to different alleles in the MHC class II locus, consistent with a pathogenic role of T lymphocytes (5).

Similar mono- and dizygotic twin concordance rates of 30 and 4%, respectively, in both MS and diabetes suggest that environmental factors trigger and/or sustain autoimmunity through interaction with the products of predisposing genes (5, 6). The search for viral triggers of autoimmunity has continued for decades. Several associations have surfaced (e.g., Refs. 7–9), but the issue is not settled in either disease (10). In addition, epidemiological surveys

identified nutritional elements as risk factors for the development of autoimmunity, specifically linking high exposure to cow milk protein (CMP) with the risk to develop MS (11–14) or autoimmune diabetes, where the available literature is more recent and more extensive (reviewed in Refs. 15–17).

Although there is controversy (18), high cow milk consumption was identified as a significant risk factor for type I diabetes (19, 20) and infants with diabetes risk-associated MHC alleles had a 13-fold higher T1DM risk when they were weaned early to cow milk-based infant formula (21). A nationwide Finnish pilot study for the international Trial to Reduce Insulin-Dependent Diabetes Mellitus in the Genetically at Risk (TRIGR) diabetes prevention effort (22) compared weaning of high-risk newborns to a nonantigenic (hydrolyzed) and a standard, cow milk-based infant formula. Although the statistical power of this pilot study was limited, infants on the hydrolyzed diet developed disease-predictive autoantibodies significantly less often than controls in a prospective, randomized, and double-blinded protocol (4 of 272 vs 24 of 284 autoantibody assays were positive in the first 2 years of life, $p < 0.001$, relative risk 5.7 (95% confidence interval, 2–16)).

Although it remains uncertain how cow milk exposure is linked to elevated risk for autoimmune disease (23), this association could lead to relatively simple avoidance trials (22). We asked whether the possible association between high cow milk exposure and MS risk suggested by epidemiological surveys years ago (11–13, 24) was associated with abnormal immunity to CMPs. During these studies, we were encouraged by the recent report of Stefferl et al. (25) that the milk protein butyrophilin can cause encephalitis in rats through antigenic mimicry with myelin oligodendrocyte glycoprotein. We found that abnormal T cell immunity to several CMPs is common in MS patients and that it is comparable to T1DM (26), but appears to involve different epitopes. In the milk protein BSA, MS patients targeted epitope BSA₁₉₃, while diabetics targeted BSA₁₅₀ (the ABBOS epitope). BSA₁₉₃ was immunogenic in SJL mice, and it induced the development of experimental autoimmune encephalitis (EAE). These observations link yet another commonly encountered dietary peptide with CNS autoimmunity,

*The Hospital For Sick Children, Research Institute, [†]Division of Neurology, St. Michael's Hospital, and [‡]Division of Laboratory Animal Services, Sunnybrook Hospital, and Departments of [§]Paediatrics and ^{||}Medicine, University of Toronto, Toronto, Ontario, Canada; and ^{||}Department of Pediatrics, Division of Endocrinology, Children's Hospital of Pittsburgh, University of Pittsburgh, Pittsburgh, PA 15260

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² S.W., I.A., and R.K.C. contributed equally to this study.

³ Address correspondence and reprint requests to Dr. Hans-Michael Dosch, The Hospital For Sick Children, Research Institute, IIIR Program, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8. E-mail address: hmdosch@sickkids.ca

⁴ Abbreviations used in this paper: MS, multiple sclerosis; CMP, cow milk protein; EAE, experimental autoimmune encephalitis; MBP, myelin basic protein; T1DM, type 1 (autoimmune) diabetes mellitus; BLG, β -lactoglobulin; SI, stimulation index.

and they extend this link to humans. There is structural homology between BSA₁₉₃ (EDKGACLLPKIE) and a portion of myelin basic protein (MBP) exon 2 (GLCHMYK) (27, 28), but although we did observe cross-reaction between the two at the level of Abs, we could not establish T cell cross-reactivity/mimicry. The nature of the endogenous protein targeted in BSA₁₉₃-immunized mice requires further study.

Materials and Methods

Human subjects

PBMC were obtained through informed consent from 48 consecutive MS patients not on steroids, Copaxone, or IFN for at least 6 mo, from 34 consecutive, newly diabetic patients, and 44 MHC-matched first-degree relatives without autoantibodies and thus a low disease risk (29, 30). HLA (DQ) typing and autoantibody measurements in diabetes patients and their relatives were done as described previously (26). Healthy adults ($n = 30$) provided population controls.

Animals

SJL/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in our vivarium. For the induction of EAE, animals 6–8 wk of age were immunized by s.c. injections of purified bovine MBP (200 μ g) (31) or of highly purified peptide (400 μ g) in CFA. Pertussis toxin, a gift from Aventis Pasteur, was injected i.v. (200 ng) at the time of immunization and 2 days later. Animals were clinically monitored daily by at least two observers, one blinded to the protocols, and EAE was scored using a standard grading system (32): 0, healthy; 1, limp tail; 2, abnormal or impaired righting reflex; 3, partial hind limb paralysis; 4, complete hind limb paralysis, and 5, moribund. Animals were sacrificed within 1 week of the initial appearance of clinical signs and perfused through the left ventricle with 40 ml of PBS followed by 10% buffered Formalin as preparation for histopathology.

Reagents

Peptides were purified >95% and confirmed by mass spectroscopy: BSA_{193–204}, EDKGACLLPKIE; BSA_{150–164}, ABBOS, FKADEKKFWG KYLYE; ICA69_{350–359}, EEGACLGPA; BSA_{394–405}, TSVFDKL KHLVD; exon 2 MBP_{71–85}, PSHARSQPLCNMYK; OVA_{152–165}, EYQDNRVSLGHFI. BSA, β -lactoglobulin (BLG), bovine casein, and OVA were purchased (Sigma, St. Louis, MO). Tetanus toxoid (TT) was a gift from Aventis Pasteur Canada.

Western blots and detection of Ab cross-reactivity

Human 18.5-kDa MBP was purified from normal adult white matter (exon 2 negative) and 21.5-kDa MBP from white matter of MS lesions (exon 2 positive) (31). Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated overnight in TBS-T buffer (TBS, 1% (v/v) Tween 20 (pH 8), and 2% blotto). Blots were probed with either affinity-purified anti-exon 2 polyclonal Ab (a gift from R. Coleman, Mount Sinai School of Medicine, New York, NY) or with affinity-purified rabbit

anti-BSA Ab raised in our laboratory. Blots were developed with Super-Signal West ECL substrate (Pierce, Rockford, IL).

T cell proliferation assays

Human PBMC (10^5 /well) were cultured in serum-free Hybrimax 2897 medium (Sigma) containing 0.1–10 μ g/ml of a given test Ag or peptide and 10 units/ml human IL-2 as described elsewhere (26). Replicate 6- to 7-day cultures were submitted to scintillation counting after an overnight pulse with [³H]thymidine. For comparison, data are presented as mean stimulation index (SI; cpm test \div cpm unstimulated cultures, the latter varied between 1 and 2000 cpm, mean 1267 ± 216). SDs of replicates were within $\pm 10\%$ of the mean. We defined a positive proliferative response to have an SI 4 SDs above mean OVA responses as described previously (26). This assay performed satisfactory in a large, blinded study of diabetes kindreds (26) and in the 1st International T Cell Workshop (33).

For murine T cell responses, SJL/J mice were immunized s.c. with 200 μ g of a given peptide emulsified in CFA (23). Draining lymph nodes were removed 9–10 days after immunization, and cells (4×10^5 /well) were cultured in AIM V serum-free medium (Life Technologies, Mississauga, Ontario, Canada) containing 0.1–10.0 μ g/well of test Ag. Cultures were pulsed overnight with [³H]thymidine on the third day of culture and submitted to liquid scintillation counting.

Statistics

Numeric results were compared by Mann-Whitney *U* or Welch tests, significance was set at 5%, and all *p* values were two-tailed. Fischer's exact test was used to analyze tables.

Results and Discussion

T cell responses to the CMPs BLG, casein, and BSA were assessed in 48 MS patients, 34 patients with recent onset T1DM, 44 of their relatives selected to have a low risk of developing diabetes, and 30 healthy controls. Nearly all 156 study subjects had tetanus-responsive T cells and these responses were similar among the groups (Fig. 1), as were responses to the T cell mitogen PHA (data not shown, two-tailed $p > 0.1$). Only one healthy control showed a small response to OVA (Fig. 1).

The median T cell responses were higher in MS patients than in healthy controls following stimulation with BLG ($p = 0.0035$), BSA ($p < 0.0001$), or casein ($p = 0.012$). Responses to CMPs were similar in patients with MS or diabetes ($p > 0.6$, Mann-Whitney *U* test, Fig. 1). Positive responses showed clear Ag dose kinetics (data not shown).

The prevalence of CMP responders was highest among MS patients (BSA, 82%; BLG, 56%; casein, 15%), followed by diabetes patients (BSA, 56%; BLG, 35%; casein, 15%) and MHC-matched first-degree relatives (BLG, 7.7%; BSA, 23%; Fig. 2). Healthy controls had only occasional responses to any of the test Ags. BSA and BLG responses were significantly more prevalent in MS or

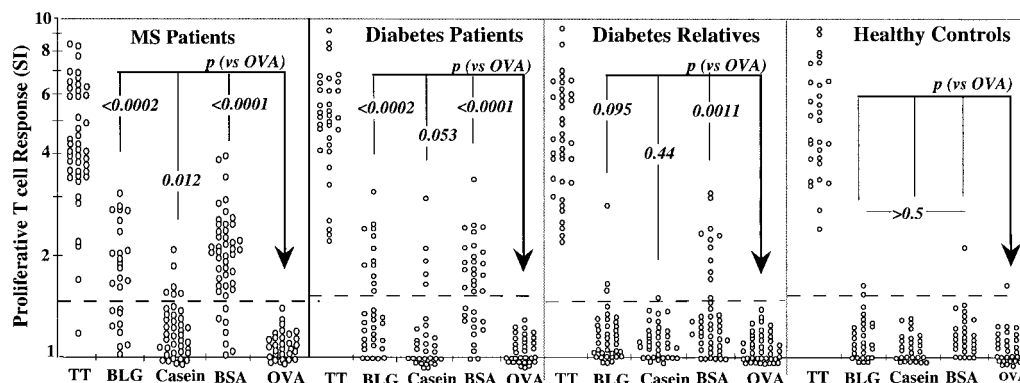


FIGURE 1. Abnormal T cell immunity to CMPs in MS and diabetes. PBMC were obtained from 48 MS patients, 34 patients with diabetes, and 44 of their MHC class II (DQ)-matched first-degree relatives selected to have a low risk of developing the disease because of absent autoantibodies. Thirty healthy subjects served as population controls. Individual proliferative responses (SI) are shown for each of the test subjects and test Ags. The dotted line indicates the cut off for positive responses, 4 SD above mean OVA responses.

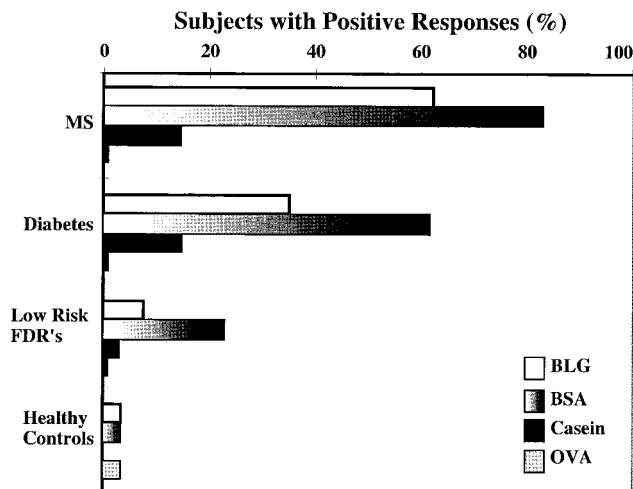


FIGURE 2. Prevalence (percent) of positive proliferative responses to the test Ags in the four study cohorts. See legend to Fig. 1 for details.

diabetes patients than in the other study cohorts ($p < 0.0001$, Fisher's exact test; Fig. 2).

These data demonstrate a common abnormality in MS T cell immunity to environmental food Ags present in cow milk but not in eggs, and they confirm similar abnormalities for diabetes (18). Since MHC-matched relatives of diabetes patients had fewer responses to BLG ($p < 0.0001$), BSA ($p = 0.0009$), and casein ($p = 0.02$), the presence of these T cells was associated with disease or disease risk and not merely with similar MHC alleles or familial predisposition (Fig. 2). Similar family studies will be attractive in MS kindreds, where they may contribute to prospective assessments of MS risk.

The diabetic immune response to BSA was mapped earlier to one major epitope, peptide BSA₁₅₀ (ABBOS, FKADEKKFWG KYLYE) (34). This peptide displays sequence homology and T cell mimicry with the Tep69 epitope of ICA69, a protein (Tep69, AFIKATGKKED) that is an autoimmune target in T1DM (23, 26) and MS (52), where it is abnormally expressed in CNS lesions (35). BSA and ICA69 share another region of considerable sequence homology: BSA₁₉₃ (EDKGACLLPKIE) and ICA69₃₅₀ (EEGACLGPA), but neither peptide is recognized in diabetics (34, 36).

We determined whether the BSA immune responses of MS patients target the ABBOS or BSA₁₉₃ epitopes (Fig. 3). Although 86% of positive BSA responses in diabetics targeted the BSA₁₅₀ (ABBOS) epitope, MS responses to BSA failed to recognize ABBOS. Instead, nearly 80% of MS BSA responses targeted the

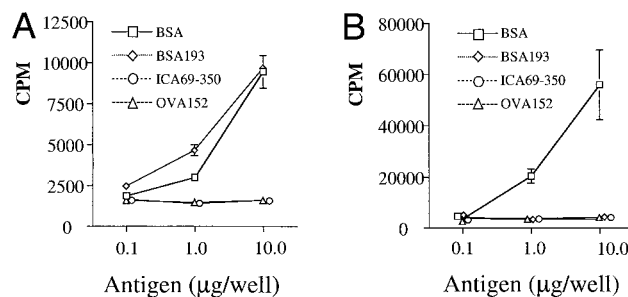


FIGURE 4. Immunity to BSA₁₉₃ and BSA in SJL/J mice. *A*, Immunization with BSA₁₉₃ generates proliferative T cell responses to BSA₁₉₃ and its protein BSA ($n = 4$). *B*, Absence of recall response to BSA₁₉₃ following immunization with BSA ($n = 4$).

BSA₁₉₃ epitope (Fig. 3), but only a minority of MS patients recognized the ICA69₃₅₀ peptide (data not shown). Although the same CMPs elicit abnormal immunity in MS and diabetes, the epitope specificity of these T cells differed.

Targeting of the ABBOS epitope by diabetic patients has its equivalent in murine T1DM, where ABBOS-reactive T cells are routinely generated (36, 37) and play a role in diabetes development (23). We decided to use in vivo experiments in mice to determine whether there was an analogous association between immunity to the environmental epitope, BSA₁₉₃, and CNS autoimmunity. We selected SJL/J mice for the following experiments, since these animals are susceptible to EAE following administration of encephalitogenic CNS Ags (38).

SJL mice immunized with BSA₁₉₃ generated T cell responses against the BSA and BSA₁₉₃ peptide, indicating that the BSA₁₉₃ peptide can be naturally generated and presented from the BSA protein in these animals (Fig. 4A). However, immunization of SJL/J mice with BSA protein failed to elicit BSA₁₉₃ recall responses, suggesting that BSA₁₉₃ is a minor, nonimmunodominant BSA epitope in SJL/J mice (Fig. 4B). Neither immunization generated responses to ICA69₃₅₀.

To determine whether the BSA₁₉₃ peptide has encephalitogenic potential, we used a standard EAE protocol (38). SJL mice were immunized s.c. with BSA₁₉₃ peptide, the OVA₁₅₂ control peptide, ICA69₃₅₀, intact BSA, or CFA only (Table I). MBP is a classic inducer of EAE and served as a positive disease control.

Of the 29 mice immunized with BSA₁₉₃ peptide, 8 developed clear clinical signs of EAE. Time to disease onset was slightly longer than in MBP-induced EAE (12.6 ± 1.7 , $n = 29$ vs 11.25 ± 0.3 , $n = 6$, $p = 0.0004$, Welch test), while maximal weight loss was similar in peptide- and MBP-induced disease (21.3 ± 6.6 , $n = 29$ vs 27.0 ± 8.0 , $n = 6$, $p = 0.16$, Welch test). The severity of the

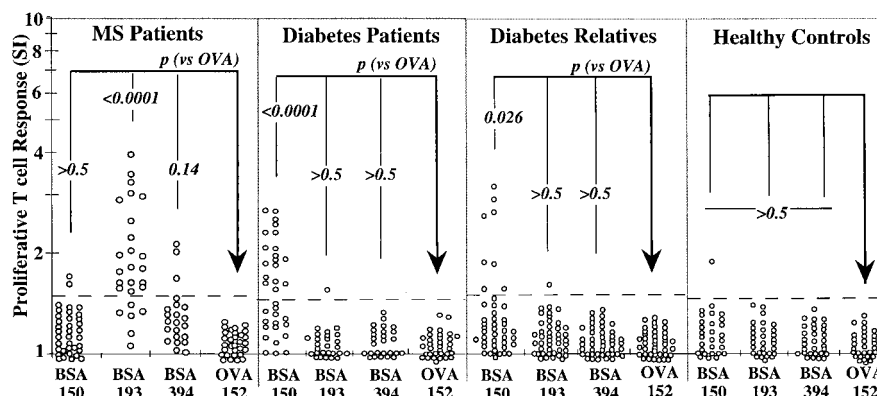


FIGURE 3. Proliferative T cell responses to the peptides indicated. See legend to Fig. 1 for description of cell donors.

Table I. Cumulative incidence of EAE for various antigens in SJL/J mice

Ag	No. with EAE	Group Score ^a (X ± SD)	EAE Score ^b (X ± SD)	Onset of EAE (day ± SD)	Maximum Weight Loss (% body weight ± SD) ^b
BSA193	8/29	0.48 ± 0.94	1.75 ± 1.03	12.6 ± 1.7	21.3 ± 6.6
MBP	5/6	2.83 ± 1.8	3.4 ± 1.34	11.25 ± 0.3	27.0 ± 8.0
BSA	0/6	NA ^c	NA	NA	5.7 ± 4.0
ICA69-350	0/6	NA	NA	NA	0
CFA Only	0/6	NA	NA	NA	0

^a Calculated from the total number of mice in each group.
^b Calculated from the number of mice that developed EAE.
^c NA, Not applicable.

disease was considerably milder in the BSA₁₉₃ group compared with MBP-injected mice ($p = 0.02$). Most (7/8) symptomatic animals immunized with BSA₁₉₃ showed ruffled fur, slowed movements, limp tails, and impaired righting reflex. Only one of these eight mice with EAE progressed to grade 4 disease with symmetric hind limb paralysis 16 days following EAE induction (Fig. 5A).

Disease induction with BSA₁₉₃ was dependent on the coadministration of pertussis toxin, since we did not observe disease in the T cell immunization experiments above. Mice injected with pertussis toxin, CFA plus OVA₁₅₂, ICA69₃₅₀, BSA, or CFA only were all clinically unremarkable. The failure to induce EAE with BSA may reflect the fact that BSA₁₉₃ is not an immunodominant

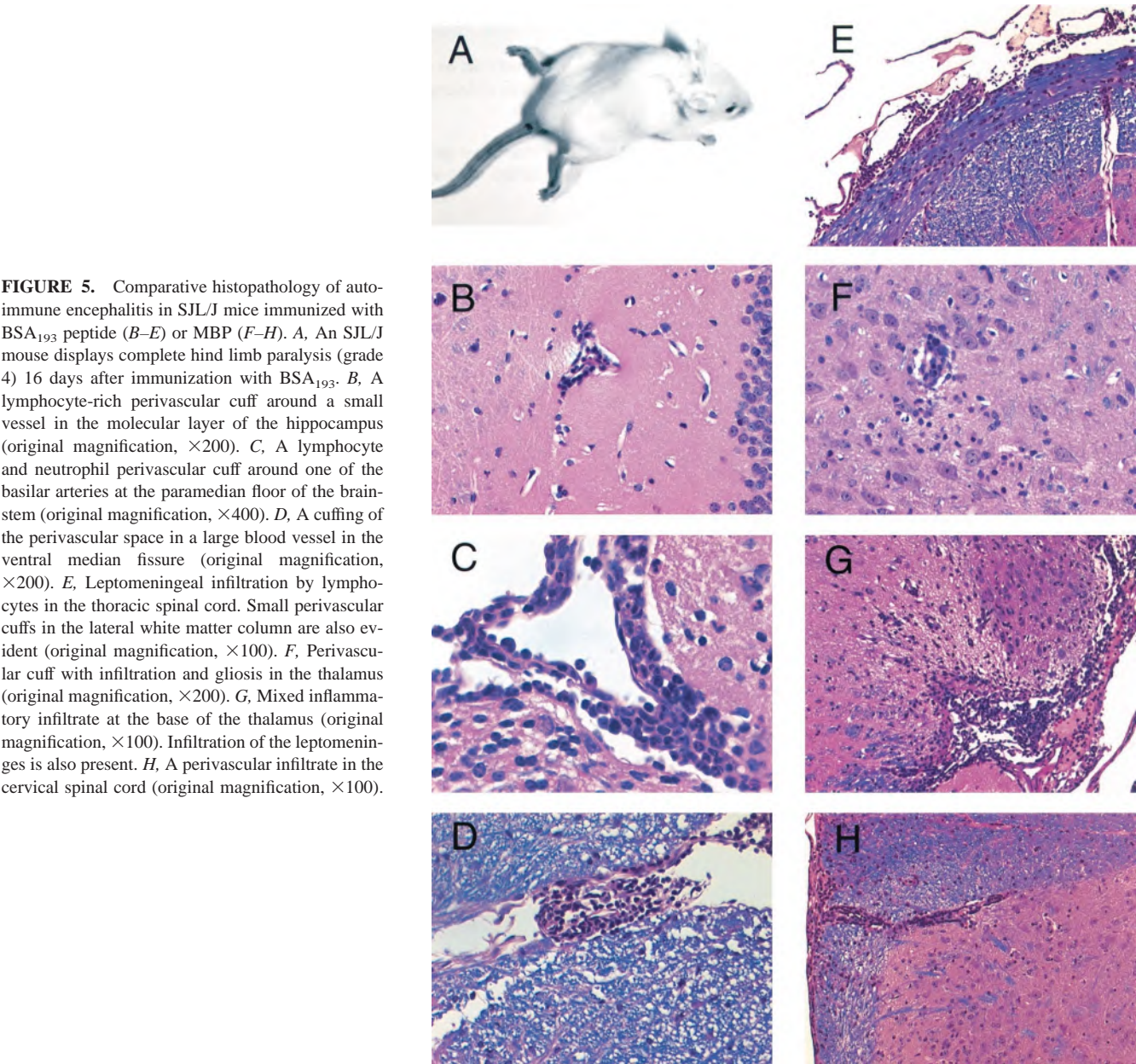


FIGURE 5. Comparative histopathology of autoimmune encephalitis in SJL/J mice immunized with BSA₁₉₃ peptide (B–E) or MBP (F–H). A, An SJL/J mouse displays complete hind limb paralysis (grade 4) 16 days after immunization with BSA₁₉₃. B, A lymphocyte-rich perivascular cuff around a small vessel in the molecular layer of the hippocampus (original magnification, ×200). C, A lymphocyte and neutrophil perivascular cuff around one of the basilar arteries at the paramedian floor of the brainstem (original magnification, ×400). D, A cuffing of the perivascular space in a large blood vessel in the ventral median fissure (original magnification, ×200). E, Leptomeningeal infiltration by lymphocytes in the thoracic spinal cord. Small perivascular cuffs in the lateral white matter column are also evident (original magnification, ×100). F, Perivascular cuff with infiltration and gliosis in the thalamus (original magnification, ×200). G, Mixed inflammatory infiltrate at the base of the thalamus (original magnification, ×100). H, A perivascular infiltrate in the cervical spinal cord (original magnification, ×100).

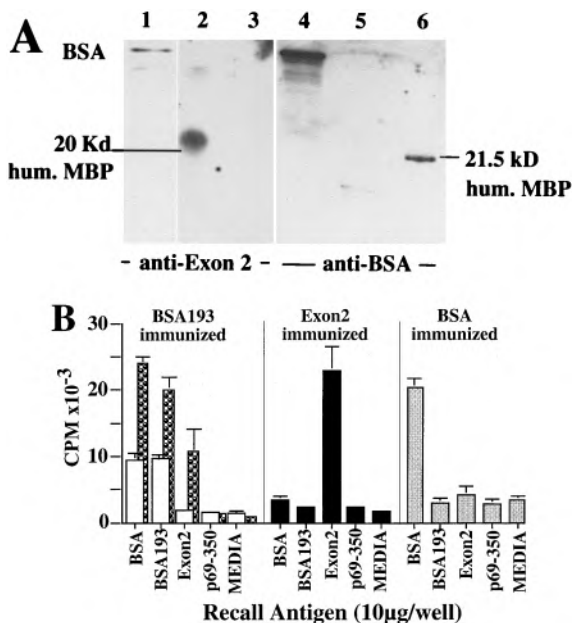


FIGURE 6. Antigenic cross-reactivity between BSA and MBP-exon 2. **A**, Abs: affinity-purified guinea pig anti-exon 2 Ab detects BSA in Western blots (lane 1) and an ~20-kDa band in white matter from an MS lesion (2 µg, lane 2), but not purified human 18.5-kDa MBP (exon 2 negative, lane 3). Affinity-purified rabbit anti-BSA Ab recognizes BSA (lane 4), recombinant human 21.5-kDa MBP (containing exon 2, lane 6), but not 18.5-kDa MBP (exon 2 negative, lane 5). Lanes from different Western blots were assembled electronically and labeled consecutively across the top. Gel loading was equalized according to molecular mass. **B**, Proliferative in vitro recall responses in SJL/J mice immunized with BSA₁₉₃ peptide ($n = 9$), MBP exon 2 ($n = 4$), or intact BSA ($n = 4$). Seven BSA₁₉₃-immunized mice failed to show antigenic mimicry to exon 2 (open bars), two additional mice with the highest BSA and peptide recall responses, also responded to exon 2 (stippled bars).

BSA epitope (Fig. 4B). This may or may not be the case after enteric protein passage; it has been suggested that enteric passage generates some peptide with preference (39).

Histopathological evaluation of brain and spinal cord sections from mice with EAE symptoms showed perivascular lymphocytic cuffing and exfiltrations in the brain, brainstem, and spinal cord (Fig. 5, B–D). Leptomeningeal infiltration by mononuclear cells was also observed in the spinal cord (Fig. 5E). The histopathology of BSA₁₉₃-injected animals was comparable, but less extensive than similar lesions in MBP-injected animals (Fig. 5, F–H). Mice immunized with OVA₁₅₂, ICA69₃₅₀, or BSA had unremarkable brain and spinal cord histology (data not shown).

These observations indicate that the BSA₁₉₃ peptide is not only targeted spontaneously by a majority of MS patients, but that it is encephalitogenic in SJL/J mice subjected to a standard EAE protocol. BSA is a ubiquitous food Ag, exposure is near universal, and one reasonable explanation for the encephalitogenic function of BSA₁₉₃ is antigenic mimicry with an unknown endogenous protein targeted by MS autoimmunity. There is considerable sequence homology with ICA69₃₅₀; however, no mimicry or EAE was observed in SJL/J mice immunized with the peptide and few MS patients recognize this peptide (data not shown).

BSA₁₉₃ also shows subtle structural homology with exon 2 of MBP (GLCHMYK). Exon 2 is a recognized target in MS autoimmunity and an exon 2 peptide can cause EAE (28, 40, 41). The expression of exon 2 is developmentally regulated through alternative splicing and largely restricted to the developing brain and to areas of myelin reconstruction, including MS lesions (27). We

found that previously described Abs to exon 2 (27) detect BSA in Western blots as well as an ~20-kDa protein in lesional MS white matter. Mass spectrographic and amino acid analysis of this band indicated that this band represents the 21.5-kDa MBP isoform, an exon 2-positive splice product associated with remyelination (data not shown). These Abs did not recognize purified exon 2-negative 18.5-kDa MBP from adult brain (Fig. 6A). In turn, affinity-purified anti-BSA Abs detected purified exon 2-positive 21.5-kDa MBP, but not the exon 2-negative 18.5-kDa isoform (Fig. 6A).

These observations may suggest antigenic cross-reactivity between BSA and exon 2 at the Ab level. Autoantibodies may play a role in autoimmune encephalitis (42–47), but the significance of the BSA-exon 2 Ab cross-reactivity in the pathogenesis of BSA₁₉₃-induced EAE remains to be determined.

T cell cross-reactivity between exon 2 and BSA₁₉₃ was inconsistent. In most animals tested (7/9), immunization with BSA₁₉₃ failed to generate mimicry responses to exon 2 (Fig. 6B, open bars), but two of nine immunized mice generated immunity to both peptides (stippled bars). Immunization with either exon 2 or full-length BSA failed to generate cross-reactive T cell responses (Fig. 6B). We are currently searching for possible endogenous ligands with BSA₁₉₃ mimicry other than exon 2.

Collectively, these observations associate MS with abnormal T cell immunity to common environmental food Ags in cow milk, and they bring to three the number of autoimmune disorders with abnormal immunity to BSA: T1DM, rheumatoid arthritis, and MS (26, 48, 49). In MS, this abnormality might contribute to the association of high cow milk exposure and the risk to develop MS or its relapses (11–13, 50). The identification of the BSA₁₉₃ epitope in MS patient experiments now permits mechanistic studies of its encephalitogenic potential in mice, analogous to similar studies in diabetes-prone animals (23, 37, 51).

Such efforts may be useful, in particular since a closer linkage of MS and abnormally enhanced immunity to CMP could lead to the design of noninvasive intervention strategies in this disease, based on a reduction of liquid cow milk exposure. Analogous efforts are under way in infants with diabetes risk (22).

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