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


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.

Environmental Peptide that Causes Encephalitis in Mice

Multiple sclerosis (MS) 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 1 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 Steffler 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 (EAEE). These observations link yet another commonly encountered dietary peptide with CNS autoimmunity.
and they extend this link to humans. There is structural homology between BSA193 (EDKGACLLPKlE) and a portion of myelin basic protein (MBP) exon 2 (GLCHYMKY) (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 3 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 μg) (31) or of highly purified peptide (400 μg) 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: BSA193–240, EDKGACLLPKlE; BSA190–245 ABBOS, FKADEKKFWG KLYE; ICA69, EGGACLGPVA; BSA194–405 TSVFDKL KHLVD; exon 2 MBP73–85, PSHARSQGLCNMYK; OVA152–165 EYQDNRYSFHLGHFL BA, 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 SuperSignal 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 μg/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 ÷ cpm unstimulated cultures, the latter varied between 1 and 2000 cpm, mean 1267 ± 216). SDs of replicates were within ±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 satisfactorily 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 μg 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 μg/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 Welsh tests, significance was set at 5%, and all p values were two-tailed. Fisher’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](image-url)

**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.
diabetes patients than in the other study cohorts \((p < 0.0001, \text{ Fisher's exact test; Fig. 2})\).

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 \(\text{(Fig. 2).} \)

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, AF11KATGKKDE) 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 (EDK1GACL1L1PKI) and ICA69 350 (EE1GACL1G1VA), 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 \(\text{(Fig. 3).} \)

86% of positive BSA responses in diabetics targeted the BSA 150 (ABBOS) epitope, while nearly 80% of MS BSA responses targeted the BSA193 epitope \(\text{(Fig. 3).} \)

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 \(\text{(Fig. 4A).} \)

Neither immunization generated responses to ICA69 350.

To determine whether the BSA193 peptide has encephalitogenic potential, we used a standard EAE protocol \((38)\).

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 \(\text{(12.6 ± 1.7, } n = 29 \text{ vs } 11.25 ± 0.3, \ n = 6, \ p = 0.0004, \text{ Welch test})\), while maximal weight loss was similar in peptide- and MBP-induced disease \(\text{(21.3 ± 6.6, } n = 29 \text{ vs } 27.0 ± 8.0, \ n = 6, \ p = 0.16, \text{ Welch test})\). The severity of the
disease was considerably milder in the BSA\textsubscript{193} group compared with MBP-injected mice ($p < 0.02$). Most (7/8) symptomatic animals immunized with BSA\textsubscript{193} 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).

<table>
<thead>
<tr>
<th>Ag</th>
<th>No. with EAE</th>
<th>Group Score\textsuperscript{a} (X ± SD)</th>
<th>EAE Score\textsuperscript{b} (X ± SD)</th>
<th>Onset of EAE (day ± SD)</th>
<th>Maximum Weight Loss (% body weight ± SD)\textsuperscript{b}</th>
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<tbody>
<tr>
<td>BSA\textsubscript{193}</td>
<td>8/29</td>
<td>0.48 ± 0.94</td>
<td>1.75 ± 1.03</td>
<td>12.6 ± 1.7</td>
<td>21.3 ± 6.6</td>
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<tr>
<td>MBP</td>
<td>5/6</td>
<td>2.83 ± 1.8</td>
<td>3.4 ± 1.34</td>
<td>11.25 ± 0.3</td>
<td>27.0 ± 8.0</td>
</tr>
<tr>
<td>BSA</td>
<td>0/6</td>
<td>NA\textsuperscript{c}</td>
<td>NA</td>
<td>NA</td>
<td>5.7 ± 4.0</td>
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<tr>
<td>ICA69-350</td>
<td>0/6</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>CFA Only</td>
<td>0/6</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Calculated from the total number of mice in each group.

\textsuperscript{b} Calculated from the number of mice that developed EAE.

\textsuperscript{c} NA, Not applicable.

Disease induction with BSA\textsubscript{193} 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 OVA\textsubscript{152}, ICA69\textsubscript{350}, BSA, or CFA only were all clinically unremarkable. The failure to induce EAE with BSA may reflect the fact that BSA\textsubscript{193} is not an immunodominant peptide.
immunized with OVA 152, ICA69 350, or BSA had unremarkable lesions of EAE analogous to similar studies in SJL/J mice subjected to a standard EAE protocol with ICA69 350; however, no mimicry or EAE was observed in SJL/J mice immunized with BSA 193 peptide (stippled bars). Immunization with either exon 2 or full-length BSA failed to generate repressive responses to exon 2 (Fig. 6B), 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 BSA 193 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 BSA 193 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 milk cow milk exposure. Analogous efforts are under way in infants with diabetes risk (22).

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References
AN ENCEPHALITOGENIC PEPTIDE IN COW MILK

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