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Differential Induction of Experimental Autoimmune Encephalomyelitis by Myelin Basic Protein Molecular Mimics in Mice Humanized for HLA-DR2 and an MBP₈₅₋₉₉-Specific T Cell Receptor

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Abstract

Multiple sclerosis (MS) is a chronic autoimmune neurological disease characterized by infiltration of peripheral inflammatory cells to the central nervous system (CNS) and demyelination of CNS white matter. Epidemiological evidence suggests a possible infectious trigger. One potential mechanism by which an infectious agent may trigger MS is via molecular mimicry wherein T cells generated against foreign epitopes cross-react with self myelin epitopes, such as myelin basic protein (MBP), with sufficient sequence similarity. It has been previously reported that an MBP₈₅₋₉₉-reactive T cell clone derived from an MS patient cross-reacted with multiple bacterial-derived mimic peptides *in vitro*. We show that the same mimic peptides can induce clinical disease in two different strains of mice transgenic for both a human MBP₈₅₋₉₉-specific TCR and HLA-DR2 (MHC II), albeit with different disease patterns – relapsing-remitting vs. monophasic. Interestingly, clinical disease correlates with CNS infiltration of CD4⁺ T cells and F4/80⁺ macrophages, but not with *in vitro* proliferative or cytokine responses of splenocytes in response to either MBP₈₅₋₉₉ or its mimics.

Keywords

autoimmunity; experimental autoimmune encephalomyelitis; molecular mimicry; myelin basic protein; multiple sclerosis; transgenic mice; T cells

1. Introduction

Multiple sclerosis (MS)¹ is a demyelinating disease of the central nervous system (CNS) of unclear etiology. More than 350,000 individuals in the United States, mostly young women,

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¹Abbreviations: CFA, complete Freund's adjuvant; Cb, cerebellum; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; LSC, lumbar spinal cord; MBP, myelin basic protein; MS, multiple sclerosis; PBS, phosphate-buffered saline; PLP, proteolipid protein; TCR, T cell receptor.

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are affected with MS [1]. Disease susceptibility in MS is associated with three MHC class II alleles in the HLA-DR2 haplotype, DRB1*1501, DRB5*0101 and DQB1*0602 [2]. The precise etiology of MS is not known, however, epidemiologic evidence suggests that some forms of MS may be triggered as a secondary consequence of infection [3]. In the genetically susceptible host, microbial infections may trigger different immunologic effector responses resulting in activation of autoreactive CD4⁺ T cells which then infiltrate the CNS and initiate the inflammatory autoimmune response [4]. There are multiple mechanisms proposed by which infections may trigger autoimmune disease including molecular mimicry, bystander activation and epitope spreading [reviewed in [5]]. Molecular mimicry can ensue when a pathogen carries an antigenic determinant that is similar enough in an amino acid sequence or structure to a self antigen that the immune response to the pathogen ‘mimic’ cross-reacts with self [6]. Peptide sequence or even structural homology is not necessarily a prerequisite for molecular mimicry [7,8]. To date, various viruses and bacteria have been linked with MS suggesting that MS could be induced by many different microbial infections. In support of this hypothesis, peptide epitopes from various viruses and bacteria have been shown to cross-activate MBP-specific T cell clones derived from MS patients in *in vitro* assays [9,10]. Identification of microbial triggers for MS may lead to prevention of disease development in genetically predisposed hosts such as first-degree relatives of MS patients carrying the HLA-DR2 haplotype by early treatment or active immunization against that specific infectious agent.

T cell clones isolated from the blood of MS patients exhibit specificity for the immunodominant CD4⁺ T cell epitope of myelin basic protein (MBP₈₅₋₉₉) [11–14]. Upon immunization with MBP₈₅₋₉₉, mice transgenic for both the human MBP₈₅₋₉₉-specific T cell receptor (TCR) and human HLA-DR2 (MHC Class II) have been reported to develop a clinical disease similar to MS and follow either a progressive or a relapsing/remitting course [15]. We employed two humanized transgenic models: one is backcrossed to the C57BL/6 background with a null mutation for mouse H2-A (dTgH2A^{neg}) [16] and the second is on a mixed DBA/2xC57BL/6 background with intact mouse MHC class II H2-A (dTgH2A^{pos}) [17]. We immunized these humanized transgenic mice with the five identified bacterial MBP₈₅₋₉₉ mimic peptides in the context of complete Freund’s adjuvant (CFA) and pertussis toxin with the goal of examining the degree of TCR functional degeneracy required for induction of molecular mimicry in the pathogenesis of MS. Specifically, we set out (1) to characterize the *in vitro* cross-reactivity to the mimics seen in the peripheral lymphoid tissue of transgenic mice, (2) to determine if priming with mimic peptides could induce disease similar to what is seen following priming with the self peptide MBP₈₅₋₉₉, and (3) to determine if the two mouse strains showed any differences in *in vitro* or *in vivo* responses to immunization with MBP₈₅₋₉₉ and/or the MBP mimic peptides which would be predictive of encephalitogenic capacity.

2. Materials and methods

2.1. Mice

Human MBP₈₅₋₉₉-specific TCR (from T cell clone Ob.1A12) and HLA-DR2 double transgenic mice backcrossed to C57BL/6 mice with a null mutation for mouse MHC class II H2-A (dTgH2A^{neg}) were provided by Dr. Vijay Kuchroo [16]. Double transgenic mice on a mixed background (DBA/2xC57BL/6) with intact mouse H2-A (dTgH2A^{pos}) were provided by Dr. Lars Fugger [17]. All mice were bred and maintained in a specific pathogen-free facility and all procedures involving animals were approved by the Northwestern University Institutional Animal Care and Use Committee.

2.2. Peptides

Peptides used to induce disease were purchased from Peptides International (GENEMED, San Francisco, CA). The amino acid composition was verified by mass spectrometry and purity was assessed by HPLC. Peptide sequences are shown in Table 1.

2.3. T cell assays

Spleens were removed from infected mice at the indicated times following priming, dissociated over wire mesh, and red cells lysed by 5 minute room temperature incubation in hypotonic buffer (17mM Tris, 140mM NH₄Cl, pH 7.2). Splenocytes were cultured in 96-well microtiter plates with indicated amounts of peptide in HL-1 medium (BioWhittaker Inc., Walkersville, Maryland, USA) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 55 µM β-mercaptoethanol (Invitrogen). Supernatant was removed from each well after 48h for cytokine measurements using the Beadlyte Mouse Multi-Cytokine Detection System (Upstate, Waltham, MA) and the Luminex 100 IS software (Luminex, Austin, TX). Plates were then pulsed with 1µCi [³H]-thymidine (MP Biomedicals, Irvine, CA) prior to the assessment of T cell proliferation after an additional 16h by liquid scintillation counting.

2.4. Disease induction

DTgH2A^{neg} mice were injected subcutaneously with 100µg peptide emulsified in CFA containing 4mg/ml *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI) distributed over three sites on the back of the mouse on day 0 and intraperitoneally with 200ng of *B. pertussis* toxin on days 0 and 2. DTgH2A^{pos} mice were injected with 250µg peptide using the same adjuvants. Peptide doses were determined by optimization experiments for each strain. Mice were scored every other day and daily when paralyzed. The following scoring scale was used: 0: normal, 1: limp tail, 2: impaired righting reflex for less than 3 seconds, 2.5: impaired righting reflex for more than 3 seconds, 3: partial hind limb paralysis of one hind limb, 3.5: partial hind limb paralysis of both hind limbs, 4: total hind limb paralysis of one hind limb, 4.5: total hind limb paralysis of both hind limbs, and 5: moribund or dead.

2.5. Immunohistochemistry

Mice were anesthetized and perfused with phosphate-buffered saline (PBS). Brains and spinal cords from each mouse were frozen in OCT (Miles Laboratories; Elkhart, IN) in liquid nitrogen. Cross-sections (10µm thick for brains and 6µm for spinal cords) from longitudinal sections of brain and spinal cord were performed. Nonspecific staining was blocked using anti-CD16/CD32 antibody (FcIII/IIR, 2.4G2; BD, PharMingen) and an avidin/biotin blocking kit (Vector Laboratories). Tissues were stained with biotin-conjugated antibody to mouse CD4, F4/80 (BD PharMingen, San Diego, CA) and mouse proteolipid protein (PLP) (AbD Serotec, Raleigh, NC). Positive staining of biotinylated antibodies was visualized by a Tyramide Signal Amplification (TSA) Direct kit (NEN, Boston, MA) according to manufacturer's instructions and fluorescein anti-mouse IgG (Vector Laboratories). Sections were counterstained with 4, 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and then coverslipped with Vectashield mounting medium (Vector Laboratories). Slides were examined and images were acquired via epifluorescence using the SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI). Sections from each group were analyzed at 40 or 100x magnification.

3. Results

3.1. Disease induction profile

Mice were primed with MBP₈₅₋₉₉ or the peptide mimics in CFA and followed for the development of clinical symptoms. In the dTgH2A^{neg} mice, priming with the majority of the peptides led to paralysis (Fig. 1a&b); the exceptions were the HINF peptide which caused no

disease in 2 of 2 experiments (data not shown), and the MBT peptide which only caused disease in one of four experiments (data not shown). Paralytic symptoms in dTgH2A^{neg} mice peaked and then remitted. In contrast, the disease course in dTgH2A^{pos} mice was remitting-relapsing (Fig. 1c&d), with relapse rates ranging between 40–100% of the mice in the different experimental groups. Interestingly, the HINF peptide was able to induce disease in this strain, but it was the weakest amongst the mimics tested. Compared to the other mimics, the HINF peptide binds to HLA-DR2 the weakest and is also a poor activator of the Ob.1A12 T cell hybridoma *in vitro* [10]. It should be noted that one dTgH2A^{pos} mouse primed with CFA and pertussis alone displayed paralysis starting at a late time point around 40 d post-priming (Fig. 1c). However, no other mouse in this or any of 2 replicate experiments displayed clinical disease.

3.2. Histology

Histological analysis was performed on primed mice at peak of initial disease (Fig. 2–5). DTgH2A^{neg} mice demonstrated CD4⁺ (Fig. 2) and F4/80⁺ (Fig. 3) cell infiltration mainly in the lumbar spinal cord of sick mice (right panels), with significant infiltration in the cerebellum only seen in mice primed with MBP₈₅₋₉₉/CFA (left panels). CD4 T cell staining seen in MBT/CFA and SAU/CFA-primed mice tended to be around the periphery of the cerebellum and not well infiltrated. Disease-free mice primed with HINF/CFA did not show cell infiltrate in these areas, demonstrating that cell infiltrate is a marker for clinical disease in this mouse model. In primed dTgH2A^{pos} mice (Figs. 4&5), slightly more cell infiltration, especially F4/80⁺ infiltrates, was seen in the cerebellum as compared with what was seen in the cerebellum of DTgH2A^{neg} mice with comparable disease scores (left panels), but again the majority of immune infiltrate was seen in the lumbar spinal cord of sick mice. Further analysis of some of the samples demonstrated infiltrate also in the hippocampus, corpus callosum, brain stem, and cervical spinal cord (data not shown). DTgH2A^{pos} mice showed significant CD4⁺ and F4/80⁺ infiltrates also during disease relapse (data not shown). There was evidence of demyelination in the LSC of both mouse strains.

3.3. T cell response to MBP₈₅₋₉₉ and the bacterially-derived peptide mimics

We next determined splenic recall responses to peptide re-stimulation of both the dTgH2A^{neg} mice and dTgH2A^{pos} mice. Previous work showed MBP₈₅₋₉₉-specific proliferation and cytokine release in naïve transgenic mice [16–18]. Here, we wanted to determine the degree of cross-reactivity of the two transgenic lines to the mimic peptides, and to determine if there was any difference between unprimed naïve mice and peptide-primed mice that may be predictive of disease onset. Splenocytes from dTgH2A^{neg} and dTgH2A^{pos} mice primed with MBP₈₅₋₉₉/CFA were taken at day 6 (prior to the onset of disease) and assessed for proliferation (Fig. 6a–d), IFN- γ release (Fig. 6e–h), IL-17 release (Fig. 6i–l), and IL-2 release (Fig. 6m–p) in response to peptide re-stimulation, and compared to responses from unprimed transgenic mice. As expected, both strains of mice showed significant proliferative responses to all the mimic peptides tested, but responses to the mimic peptides tended to be slightly lower than the response to self MBP₈₅₋₉₉. Interestingly, the mice responded most robustly to the SAU mimic and least robustly to the HINF and BACS mimics, which correlates well with the *in vitro* proliferation pattern previously reported for the human parental Ob.1A12 T cell clone [10]. In contrast to the pattern of proliferation, the amount of cytokine released in response to the mimic peptides was in general much lower than the amount of cytokine released in response to the self peptide. Cytokine production has been found to be dependent on the strength of signal delivered to the T cell [19], so the mimics may deliver a signal that is below a critical threshold required for robust cytokine production/release. In addition, there was no difference between primed and naïve mice either in degree of proliferation or degree or type of cytokine released. Thus, splenic responses to peptide did not correlate well with disease development. However, in general the lowest amounts of IFN- γ and IL-17 at the 10 μ M peptide

dose were induced in unprimed T cells by the mimic peptides which were the least efficient at inducing disease: MBT and HINF for the dTgH2A^{neg} mice, and HINF for the dTgH2A^{pos} strain.

4. Discussion

Our results are in agreement with previous studies showing that immunization with the myelin-derived MBP₈₅₋₉₉ peptide induces clinical disease in two different strains of mice transgenic for a human MBP-specific TCR and the human MHC HLA-DR2 molecule [16,17]. In this paper, we set out to further characterize this model by determining if disease could also be caused by immunization with bacterially-derived peptide mimics of the self MBP peptide. With only one exception, all mimic peptides were able to cause robust paralytic disease when injected with CFA and pertussis toxin. This is in contrast to what we found in an SJL model of molecular mimicry employing PLP₁₃₉₋₁₅₁ mimic peptides, where priming with the mimics in CFA failed to cause paralytic disease in spite of their ability to induce T cells cross-reactive to a myelin epitope [20–22]. In both the humanized transgenic and SJL models, the corresponding mimics were weaker than the self-peptide in their MHC binding and/or the magnitude to which they could activate self-specific T cells *in vitro* [10,22]. The ability of the mimics to cause disease in the humanized transgenic models is likely related to the extremely high precursor frequency of myelin-specific T cells in the transgenic models as compared with wild type SJL mice. A higher precursor frequency may more easily allow for the activation of a threshold amount of T cells necessary to cross the blood-brain barrier and initiate paralytic disease. Similar to what we saw in the SJL model, splenocyte proliferation and cytokine release in response to peptide was not a good predictor of future disease induction, but presence of CNS infiltrate correlated well with clinical symptoms. We are currently examining the frequency of cytokine-producing T cells (IFN- γ and IL-17) which have infiltrated the CNS in the mimic peptide-primed mice to determine if a better correlation with disease-inducing ability is observed.

One of the most significant and interesting findings emerging from these studies is the difference in clinical disease manifested by the two different strains of humanized transgenic mice employed. In our hands, dTgH2A^{pos} mice developed a relapsing-remitting course of paralysis upon peptide priming, while the disease in dTgH2A^{neg} mice was monophasic. Histological characteristics were also somewhat different between the two strains of mice, with brain infiltration being more somewhat prevalent in mimic-primed dTgH2A^{pos} mice than mimic-primed TgH2A^{neg} mice. Thus, even though both transgenic strains were made using the same MBP₈₅₋₉₉-specific TCR and MHC cosmids, the background characteristics of the mice are sufficiently disparate to cause very different clinical pictures. It is unlikely that the relapsing disease observed in the dTgH2A^{pos} mice is due to presentation and activation of Th1/Th17 cells by the residual H-2 I-A^b molecules as it was previously reported that mice that expressed the humanized TCR (and not the humanized MHC) did not develop disease when primed with MBP₈₅₋₉₉ peptide and their splenic T cells did not respond to peptide, nor did peptide-primed dTgH2A^{neg} mice develop disease [16,17]. We are currently determining the response of wildtype C57BL/6 mice to priming with the various peptide mimics. Two documented differences between these strains are 1) the dTgH2A^{neg} mice have a null mutation in the mouse MHC class II molecule H2-A, while dTgH2A^{pos} mice still express the mouse MHC Class II, and 2) the dTgH2A^{neg} mice are completely backcrossed onto a C57/BL6 background, while dTgH2A^{pos} mice are on a mixed DBA/2xC57BL/6 background. Both of these differences could likely produce differences in the endogenous murine T cell repertoire that could in turn result in the different clinical outcomes. For example, the absence of relapses in primed dTgH2A^{neg} mice may be due to the presence of regulatory T cells capable of controlling MBP₈₅₋₉₉-specific T cell responses. We currently exploring this possibility and otherwise characterizing the immunologic differences between the two strains that would account for the different clinical manifestations.

Some of the mimic peptides are epitopes of bacteria that are part of the human flora and frequent pathogens in humans (*e.g.*, *S. aureus*). Others such as *M. tuberculosis* are seen more in certain geographic areas which are not necessarily areas with increased prevalence of MS. Thus, a better approach to study molecular mimicry-induced CNS autoimmunity is likely to be in the context of infection by these pathogens as we have previously suggested [21].

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Fig. 1.

Priming with MBP₈₅₋₉₉ and its mimics causes monophasic disease in dTgH2A^{neg} mice and relapsing-remitting disease in dTgH2A^{pos} mice. **(a) & (b)** DTgH2A^{neg} mice were primed with 100µg peptide emulsified in CFA on d0, given 200ng of *B. pertussis* toxin i.p. on days 0 and 2, and followed for the presence of clinical symptoms. The following scoring scale was used: 0: normal, 1: limp tail, 2: impaired righting reflex for less than 3 seconds, 2.5: impaired righting reflex for more than 3 seconds, 3: partial hind limb paralysis of one hind limb, 3.5: partial hind limb paralysis of both hind limbs, 4: total hind limb paralysis of one hind limb, 4.5: total hind limb paralysis of both hind limbs, and 5: moribund or dead. Shown for each experimental group is the incidence and rate of relapse (for mice with initial disease). **(c) & (d)** DTgH2A^{pos} mice were primed with 250µg peptide emulsified in CFA on d0, given 200ng of *B. pertussis* toxin i.p. on days 0 and 2, and followed for the presence of clinical symptoms. Results shown are representative of 1–3 replicate experiments. Peptide sequences and abbreviations are found in Table 1. Results are representative of at least two separate experiments.

Fig. 2.

Immunohistochemical analysis of CD4⁺ infiltrates in primed dTgH2A^{neg} mice at peak of disease. Mice were primed with 100µg peptide emulsified in CFA on d0, given 200ng of *B. pertussis* toxin i.p. on days 0 and 2, and followed for the presence of clinical symptoms. Brain and spinal cord sections were taken from peptide-primed mice at peak of initial disease (before day 20) and stained with antibody to CD4 (red) and PLP (green), and co-stained with DAPI (blue). Sections from cerebellum (Cb) are shown in left panels and sections from lumbar spinal cord (LSC) are shown in right panels. Results shown are representative of replicate staining. Peptide sequences and abbreviations are found in Table 1.

Fig. 3.

Immunohistochemical analysis of F4/80⁺ infiltrates in primed dTgH2A^{neg} mice at peak of disease. Mice were primed with 100μg peptide emulsified in CFA on d0, given 200ng of *B. pertussis* toxin i.p. on days 0 and 2, and followed for the presence of clinical symptoms. Staining was performed as described in Fig. 2.

Fig. 4.

Immunohistochemical analysis of CD4⁺ infiltrates in primed dTgH2A^{pos} mice at peak of disease. Mice were primed with 250μg peptide emulsified in CFA on d0, given 200ng of *B. pertussis* toxin i.p. on days 0 and 2, and followed for the presence of clinical symptoms. Staining was performed as described in Fig. 2.

Fig. 5. Immunohistochemical analysis of F4/80⁺ infiltrates in primed dTgH2A^{Pos} mice at peak of disease. Mice were primed with 250μg peptide emulsified in CFA on d0, given 200ng of *B. pertussis* toxin i.p. on days 0 and 2, and followed for the presence of clinical symptoms. Staining was performed as described in Fig. 2.

Fig. 6.

Cross-reactive T cell proliferative and pro-inflammatory cytokine profiles of unprimed and MBP₈₅₋₉₉-primed transgenic mice. Mice ($n = 2/\text{group}$) were primed on day 0 with 100 μg peptide (dTgH2A^{neg} mice) or 250 μg peptide (dTgH2A^{pos} mice) in CFA or left unprimed. On day 6, splenocytes were cultured in 96-well microtiter plates (5×10^5 cells/well) with the indicated concentrations of peptide or media alone (no pep). Proliferative responses (a–d) were determined by ³H-TdR incorporation (counts per minute, or CPM) after 72 h in culture. Prior to pulsing with ³H-TdR, supernatants from triplicate wells were taken at 48 h, pooled and analyzed for levels of (e–h) IFN-γ, (i–l) IL-17, and (m–p) IL-2 by Liquechip. Results are representative of 3–4 separate experiments.

Peptide sequences of MBP₈₅₋₉₉ and its bacterial mimics
Boldface indicates residues shared with the self myelin epitope. Residues 5 and 8 are primary MHC contact residues; residues 6, 7 and 9 are primary TCR contact residues [10].

Peptide source (abbreviation)	Amino acid residue/sequence														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Myelin Basic Protein (MBP)	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R
<i>Staphylococcus aureus</i> (SAU)	V	L	A	R	L	H	F	Y	R	N	D	V	H	K	E
<i>Mycobacterium avium</i> (MBA)	Q	R	C	R	V	H	F	L	R	N	V	L	A	Q	V
<i>Mycobacterium tuberculosis</i> (MBT)	Q	R	C	R	V	H	F	M	R	N	L	Y	T	A	V
<i>Bacillus subtilis</i> (BACS)	A	L	A	V	L	H	F	Y	P	D	K	G	A	K	N
<i>Haemophilus influenzae</i> (HINF)	D	F	A	R	V	H	F	I	S	A	L	H	G	S	G