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Genetic Analysis of the Influence of Neuroantigen-Complete Freund's Adjuvant Emulsion Structures on the Sexual Dimorphism and Susceptibility to Experimental Allergic Encephalomyelitis

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The induction of organ-specific autoimmune diseases, such as experimental allergic encephalomyelitis (EAE) the principal animal model of multiple sclerosis (MS), relies on the use of complete Freund's adjuvant (CFA) emulsions. In this study we report that the physical structure of the particles comprising neuroantigen-CFA emulsions significantly influences the genetic control of the incidence and sexual dimorphism seen in EAE. Immunization of (B10.S/SgMcdJ × SJL/J) F_2 mice segregating the quantitative trait loci (QTL) controlling EAE in susceptible SJL/J and resistant B10.S/SgMcdJ mice with emulsions consisting of particles where the *Mycobacterium tuberculosis* and neuroantigens are localized on the phase surfaces led to severe EAE in 98.8% of the mice, overriding all sex-specific and non-sex-specific genetic checkpoints. In contrast, F_2 mice immunized with emulsions where the bacterial products and encephalitogens are buried inside the water/oil vesicles exhibited a significant reduction in disease incidence (7.5%) and a sexual dimorphism (5% male *versus* 10% female). A genome scan identified QTL on chromosomes 7 and 11 controlling the sexual dimorphism as a function of the physical structure of the emulsion. The chromosome 11 QTL co-localizes with *eae6b*, and with *Il12b* and heptatitis A virus cellular receptor 2 (*Havcr2*, formerly known as *Timd3*), both of which are candidate genes for this QTL. Sequence analysis of the SJL/J and B10.S/SgMcdJ alleles indicates that both gene products are structurally monomorphic. Expression analysis also excluded both as candidates for this sex-specific QTL. These results reinforce the importance of gene-environment interactions in initiating and propagating autoimmune disease of the central nervous system, particularly in the context of susceptibility to MS and disease heterogeneity. *(Am J Pathol 2003, 163:1623–1632)*

The use of complete Freund's adjuvant (CFA) to induce organ-specific autoimmune disease dates back to 1947 when Freund reported the induction of experimental allergic encephalomyelitis (EAE) in guinea pigs using a single injection of central nervous system (CNS) antigens emulsified in CFA.[1] Before this time, multiple injections without adjuvants were used.[2] Subsequently, the use of CFA resulted in eliciting a plethora of experimental models of organ-specific autoimmune disease in a wide variety of species[3] including humans.[4,5] The initial theories put forth by Freund to explain the adjuvanticity of CFA centered around three mechanisms: 1) persistence of the antigen at the site of injection, 2) transport of the antigen to the lymphatic system, and 3) development of sensitization.[6] These hypotheses served as the impetus for the studies establishing that autoantigen sensitization using CFA emulsions preferentially stimulates delayed-type hypersensitivity reactions mediated by $CD4^+$ Th_1 effector T cells.[3]

Additional adjuvants, such as *Bordetella pertussis*[7,8] and pertussis toxin (PTX),[9,10] the major exotoxin produced by *B. pertussis*,[11] were also found to be useful for inducing certain diseases, particularly in rats and mice. However, *B. pertussis* and PTX elicit both strong DTH and anaphylactic antibody responses.[12] With respect to disease induction, it was recently demonstrated that the histamine H_1 receptor underlies *Bphs*, a shared autoimmune disease susceptibility locus between EAE and autoimmune orchitis, associated with the adjuvanticity of PTX and controlling the development of pathogenic $CD4^+$ Th_1 effector T cells.[13–15]

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We recently demonstrated that PTX also overrides many of the genetic checkpoints identified in mapping studies where CFA was used as the sole adjuvant.[16] Additionally, the use of PTX as an ancillary adjuvant resulted in less severe disease in female (B10.S/DvTe × SJL/J) × B10.S/DvTe backcross mice compared to female backcross mice immunized without PTX. In this same study, comparative mapping studies using backcross mice immunized with and without PTX led to the identification of adjuvant-dependent, sex-specific QTL.[16] These results suggest that the sexual dimorphism seen in EAE may be due in part to sex-specific QTL controlling responsiveness to the adjuvants used to induce disease. We report here that immunization of (B10.S/SgMcdJ × SJL/J) F_2 mice with CFA emulsions consisting of particles where the *Mycobacterium tuberculosis* and encephalitogens are localized on the phase surfaces do not exhibit a sexual dimorphism. In contrast, F_2 mice immunized with emulsions where the bacteria and neuroantigens are buried inside the water/oil vesicles exhibit a significant sexual dimorphism (5% male *versus* 10% female) and that QTL on chromosomes 7 and 11 control susceptibility to sexually dimorphic EAE as a function of the particulate structure of neuroantigen-CFA emulsions.

Materials and Methods

Animals

Male and female SJL/J and B10.S/SgMcdJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Retired male and female SJL/J breeders were purchased from Harlan (Indianapolis, IN). Male and female (B10.S/SgMcdJ × SJL/J) F_1 hybrid animals were generated and (B10.S/SgMcdJ × SJL/J) F_2 mice were produced by intercrossing F_1 animals. The same parental and F_1 hybrid mice were used to generate all F_2 mice used in this study. F_1 hybrid and F_2 intercross mice were kept in the same room within the vivarium at the College of Veterinary Medicine at the University of Illinois, Urbana-Champaign (Urbana, IL). Animals were maintained in accordance with the Animal Welfare Act and the Public Health Service Policy on the Humane Care and Use of Laboratory Animals.

Mouse Spinal Cord Homogenate (MSCH)

MSCH was generated using retired breeder SJL/J mice purchased from The Jackson Laboratory. Spinal cords were extracted by insufflation, mixed with an equal volume of distilled H_2O, homogenized in a Pyrex tissue homogenizer, and filtered through nylon mesh. The MSCH was lyophilized overnight and resuspended at 50 mg/ml in phosphate-buffered saline (PBS) and 1-ml aliquots stored at −70°C until used. The same pool of SJL/J MSCH was used for all injections in this study.

Preparation of MSCH-CFA Emulsions

MSCH-CFA emulsions with the *Mycobacterium tuberculosis* and encephalitogens localized on the external surface phase (ext-ENC-CFA) or buried within the water/oil vesicles (int-ENC-CFA) were prepared by high-speed homogenization and syringe extrusion, respectively. Homogenized emulsions were prepared by adding equal volumes of CFA (IFA supplemented with 0.1 mg/ml *M. tuberculosis*, H37Ra, as described above) and SJL/J MSCH in PBS into a 20-ml homogenization chamber fitted to a high-speed Waring blender. Homogenization was performed at room temperature for 1 minute. Syringe extruded MSCH-CFA emulsions were prepared using disposable syringes and a 21-gauge double-hub microemulsifying needle. The oil and water phases were emulsified at a 1:1 ratio. One syringe contained incomplete Freund's adjuvant (Sigma, St. Louis, MO) supplemented with 0.1 mg/ml *M. tuberculosis*, H37Ra (Difco Laboratories, Detroit, MI). The other syringe contained an equal volume of SJL/J MSCH in PBS at a concentration of 6.67 mg/ml. Both syringes were cleared of trapped air, affixed to the microemulsifying needle, and the plunger of the syringe containing the aqueous phase was first depressed to extrude the entire contents into the oil phase. Subsequently, the plungers were depressed alternately until a non-separating emulsion was achieved. Both emulsions were non-separating on a water surface.

Induction and Evaluation of EAE

Mice were immunized for the induction of EAE as previously described.[17] Briefly, they were inoculated with 0.3 ml of SJL/J MSCH-CFA emulsion via two subcutaneous injections in the posterior right and left flank (2 × 0.15 ml). One week later all mice were similarly injected at two sites on the right and left flanks anterior of the initial injection sites. In this way, each animal received a total of 2.0 mg dry weight SJL/J MSCH and 30.0 μg of *M. tuberculosis* H37Ra. A cohort of B10.S/SgMcdJ mice were immunized with proteolipid protein peptide 139–151 (HSLGKWLGHPDKF) ($PLP_{139-151}$) emulsified in CFA by injecting 0.2 ml of emulsion made by mixing equal volumes of 1 mg/ml PLP in PBS and 4 mg/ml *M. tuberculosis* H37Ra. Twenty-four hours later each mouse received by i.v. injection 200 ng PTX (List Biological Laboratories, Inc., Campbell, CA).

Mice were weighed and scored for clinical signs of disease daily from day 10 post-injection through day 60. Severity scores ranging from 0 to 4 were assigned as previously described[18]: 0, no clinical signs; 1, flaccid tail without hind leg weakness; 2, flaccid tail with hind limb weakness; 3, complete hind limb paralysis and floppy tail; 4, hind leg paralysis accompanied by a floppy tail and urinary or fecal incontinence; 5, quadriplegia and/or death. The incidence of EAE was recorded as positive for any mouse exhibiting clinical signs for one or more days. Susceptibility was also analyzed as a quantitative trait, using a disease index generated by averaging the clinical score for each animal over the course of the experiment. Severity of disease among affected animals was

analyzed using the single high score for each mouse exhibiting clinical signs and onset was the day clinical signs were first observed. A weight index was calculated as the average weight change from the preinjection baseline to the peak of clinical disease for the population.

Genotyping

Genomic DNA was isolated from individual liver samples as previously described.[14] Microsatellite markers were purchased from Research Genetics (Huntsville, AL), or synthesized according to published sequences (http://www-genome.wi.mit.edu/cgi-bin/mouse/index). Polymerase chain reaction (PCR) parameters for microsatellite typing were previously described.[17] Microsatellite size variants were resolved by electrophoresis on large-format denaturing polyacrylamide gels, and visualized by autoradiography.

Linkage Analysis

Thirty-seven of 494 (B10.S/SgMcdJ $\times$ SJL/J) F_2 mice immunized with int-ENC-CFA emulsions exhibited clinical signs of EAE. A set of 39 randomly selected sex-matched littermates served as the unaffected population for linkage analysis. Linkage analysis was performed using information derived from a genome scan with 127 informative microsatellite markers covering all 19 autosomes and the X-chromosome. Linkage maps were generated using the Kosambi mapping function in the MAPMAKER/EXP computer package.[19,20]

QTL analysis was performed using the disease index (susceptibility) and severity or high score. Composite interval mapping (CIM) was carried out using model 6 of the Zmapqtl program in QTL Cartographer v1.30 software package (http://statgen.ncsu.edu/qtlcart/cartographer.html). By combining classical interval mapping with multiple regression analysis, CIM allows for more precise QTL localization than classical interval mapping. Additionally, CIM controls for spurious ghost loci.[21–22] Significant markers are first chosen using a linear regression model with a forward/backward selection procedure in the SRmapqtl module of QTL Cartographer. Markers flanking the test interval are added to the regression model to control for the presence of linked QTL. Additional markers, unlinked to the interval, but with significant effects on the trait are added to the model to control for the genetic background. Two cM increments with a window size of 10 cM and all 9 significant background markers selected via SRmapqtl were used in our CIM analyses. Tests of significant linkage are reported as likelihood ratio test (LRT) statistics. Significance of the linkage between marker loci and putative QTL was assessed by permutation-based threshold analysis.[23,24] Significant ($\alpha = 0.05$) and suggestive ($\alpha = 0.10$) experiment-wise critical values were determined using the distribution of maximum LRT statistics from 1000 permutations of our data. Analysis of linkage stratified by sex was carried out using QTManager (http://mapmgr.roswellpark.org/mapmgr.html).

Havcr2/Timd3 Allotyping

DNA samples from a number of inbred strains were purchased from The Jackson Laboratory or were prepared from tissues on hand, as described above. We designed two sets of oligonucleotide primers that amplify two portions of the *Havcr2/Timd3* genomic sequence: one set for a positive control amplification (*Havcr2/Timd3* control), and one set that flanked a microsatellite region (*Havcr2/Timd3* MS) between nucleotides 1686 and 1862 in the 3′-UTR of the AKR/J mouse *Havcr2/Timd3* sequence (AF450241): *Havcr2/Timd3* MS forward primer: 5′-AGCCTAGG TGCTGAGTTCCA-3′, *Havcr2/Timd3* MS reverse: 5′-GAAGGTTCTCTCTGTGCCTGC-3′. *Havcr2/Timd3* control forward primer: 5′-CTACCTACATCTGGGACACTTG-3′, reverse: 5′-GACTCTGGATGACC ATGGGAC-3′. Fragments amplified by radiolabeled primer pairs were run on polyacrylamide gels as above, and *Havcr2/Timd3* alleles read directly from the autoradiographic image. The absence of an *AflIII* restriction site in the PCR product amplified by the control primers distinguished DBA/2J (*d*) or AKR/J (*a*) *Havcr2/Timd3* alleles.[25] The results were combined into unique haplotypes representing the combination of alleles at each of these *Havcr2/Timd3* sequence characteristics.

Nucleic Acid Sequencing

Total RNA was isolated from the spleen of adult B10.S/SgMcdJ and SJL/J mice using TRIzol Reagent (Invitrogen, Carlsbad, CA). Reverse transcription (RT)-PCR was performed to obtain cDNA. First-strand cDNA was synthesized by reverse transcription of 1.0 μg total RNA primed with poly (dT_{16}) oligonucleotide and Superscript^{II} reverse transcriptase (Invitrogen). cDNAs were PCR-amplified using *Taq* polymerase and *Havcr2/Timd3* specific primer pairs (Operon, Alameda, CA) flanking the mRNA coding regions (Forward: 5′-CTGCTGCTGC TGCAACTACTA-3′ and reverse: 5′-GGACTGCCACTTTTAAAGGCT-3′). The amplified fragments were cloned and PCR-screened. Insert-positive plasmid DNAs were sequenced using vector primers and the ABI PRIZM Dye Terminator Reading Reaction Cycle Sequencing Kit on a model 373A automated DNA sequencer (PerkinElmer, Applied Biosystems Division, Foster City, CA). PCR fragments were sequenced from both insert termini.

$CD4^+$ T-Cell Preparation and Activation

$CD4^+$ T cells were isolated from spleen and lymph nodes of adult B10.S/SgMcdJ and SJL/J mice by negative selection as described[26] using a combination of anti-MHC class II (m5/115), anti-CD8 (TIB105), anti-NK1.1, and anti-Mac1 mAbs (PharMingen, San Diego, CA). Cells were activated with plate-bound anti-CD3 (145–2C11, 5 μg/ml) and soluble anti-CD28 (1 μg/ml) mAbs (PharMingen) in the presence or absence of IFNγ (R&D Systems Inc., Minneapolis, MN) at a final concentration of 200 U/ml for 48 hours.

Real-Time PCR Analysis of Gene Expression

Total RNA was extracted from non-activated and activated CD4$^+$ T cells using Ultraspec RNA Isolation Reagent (Biotecx Laboratory, Houston, TX) as recommended by the manufacturer. 1.0 μg of total RNA was used as a template to synthesize first-strand cDNA using random primers (Promega, Madison, WI) with Superscript^{II} reverse transcriptase (Invitrogen). Real-time quantitative RT-PCR was performed using the TaqMan system (Applied Biosystems). PCRs were performed using the TaqMan Universal PCR Master Mix and the ABI PRISM 7700 Sequence Detection System. *Havcr2/Timd3*, *Il12b* and γ-actin (*Actg*) (internal reference) were measured by real-time PCR using probes labeled with 6-carboxyfluorescein (FAM) and Black Hole Quencher (BHQ). The *Havcr2/Timd3* and *Il12b* probes and primer set sequences used were as previously described.[27,28] The *Actg* probe and primer sequences were: probe, 5′-6-FAM-d(CATTGCTCCCCCTGAGCGCAA)-BHQ-1–3′; forward primer, 5′-d(GCACCTAGCACGAT GAAGATTAA GA)-3′; reverse primer, 5′-d(AGCCACCGATCCAGACT-GAGT)-3′ (Biosearch Technologies, Novato, CA). *Havcr2/Timd3*, *IL12b*, and *Actg* mRNA levels were analyzed separately using the standard curve method. A no-RT control was also prepared for each sample and no genomic DNA was detected. As a positive control for Havcr2/Timd3 expression, cDNA was prepared from Th1-skewed CD4$^+$ T cells. Briefly, CD4$^+$ T cells were stimulated with anti-CD3 and anti-CD28 mAbs in the presence of IL-12 (3.5 ng/ml; R&D Systems) and IFNγ (200 U/ml) for 4 days. For IL12β, cDNA was prepared from the spleens of mice immunized with CFA + PTX taken at 3 days post-injection. Total RNA was isolated and reverse-transcribed into first-strand cDNA. Varying amounts of the positive control cDNA were used to determine the linear range of the PCR reactions. 1 μl of the sample cDNA was used in the PCR reaction and the comparative threshold cycle (C_T) value obtained was used to calculate the amount of sample cDNA required to reach the linear range established by the standard. The values obtained from separate reactions were averaged and the ratio of Havcr2/Timd3:Actg and Il12β:Actg calculated. For each sample, the Havcr2/Timd3 and Il12β C_T value was expressed as an *n*-fold difference compared to the unstimulated B10.S/SgMcdJ sample.

Table 1. Incidence of EAE in Mice Immunized with Int-ENC-CFA and Ext-ENC-CFA Emulsions

Strain	Int-ENC-CFA	Ext-ENC-CFA	*P* value
SJL/J (5–6 weeks)			
Female	4/5	5/5	
Male	0/5	4/5	
Total	4/10	9/10	0.019
SJL/J (>24 weeks)			
Female	4/5	5/5	
Male	4/5	5/5	
Total	8/10	10/10	
B10.S/SgMcdJ			
Female	0/5	5/5	
Male	0/5	5/5	

Animals were scored as unaffected or affected (any clinical signs) by D30 post-injection.

Results

Cohorts of SJL/J, B10.S/SgMcdJ, (B10.S/SgMcdJ × SJL/J) F_1 hybrid and (B10.S/SgMcdJ × SJL/J) F_2 intercross mice were immunized under the same conditions and at the same sites with identical preparations of SJL/J MSCH-CFA emulsions with the exception of the distribution of the bacterial products and encephalitogens within the particles comprising the emulsions. F_1 hybrid and F_2 intercross mice were matched for parents, sex, age, and season. The incidence of EAE was significantly greater in animals immunized with ext-ENC-CFA emulsions compared to mice immunized with int-ENC-CFA emulsions (Table 1). Similar results were obtained with B10.S/SgMcdJ mice immunized with ext-$PLP_{139-151}$-CFA (9 of 10 mice sick) and int-$PLP_{139-151}$-CFA (1 of 10 mice sick) emulsions.

All (B10.S/SgMcdJ × SJL/J) F_1 hybrid mice (24 of 24) and nearly all (B10.S/SgMcdJ × SJL/J) F_2 intercross mice (248 of 251) immunized with ext-ENC-CFA developed EAE (Table 2). In contrast, only 22% (11 of 50) of

Table 2. Clinical Signs Associated with EAE in (B10.S/SgMcdJ × SJL/J) F_1 Hybrid and F_2 Intercross Mice Injected with Ext-ENC-CFA and Int-ENC-CFA Emulsions

Trait	F_1 hybrid	*P* value	F_2 intercross	*P* value
Incidence				
Ext-ENC-CFA	24/24 (100)*		248/251 (99)	
Int-ENC-CFA	11/50 (22)	<0.0001	37/494 (8)	<0.0001
Onset				
Ext-ENC-CFA	15.4 ± 1.4		15.4 ± 4.4	
Int-ENC-CFA	23.6 ± 12.0	0.0021	21.1 ± 7.8	<0.0001
Severity				
Ext-ENC-CFA	3.8 ± 0.4		3.1 ± 1.8	
Int-ENC-CFA	2.3 ± 0.8	<0.0001	2.3 ± 0.9	0.0086
Weight loss†				
Ext-ENC-CFA	4.6 ± 2.4		2.5 ± 2.5	
Int-ENC-CFA	0.1 ± 2.6	<0.0001	0.9 ± 1.6	<0.0001

*Percent affected.
†The average weight loss was calculated using the total loss in grams for each animal from the baseline weight at injection to the peak of clinical disease.

Table 3. Clinical Signs Associated with EAE in Female and Male (B10.S/SgMcdJ × SJL/J) F_1 Hybrid and F_2 Intercross Mice Injected with Ext-ENC-CFA and Int-ENC-CFA Emulsions

Trait	F_1 hybrid	*P* value	F_2 intercross	*P* value
Incidence				
Ext-ENC-CFA				
Female	12/12 (100)*		125/127 (98)	
Male	12/12 (100)		123/124 (99)	
Int-ENC-CFA				
Female	7/26 (27)		24/233 (10)	
Male	4/24 (17)		13/261 (5)	0.025
Onset				
Ext-ENC-CFA				
Female	16.0 ± 1.5		15.4 ± 4.5	
Male	14.8 ± 1.1		15.3 ± 4.4	
Int-ENC-CFA				
Female	24.9 ± 14.9		22.1 ± 8.3	
Male	21.0 ± 4.6		19.1 ± 6.7	
Severity				
Ext-ENC-CFA				
Female	3.7 ± 0.5		2.9 ± 1.2	
Male	3.9 ± 0.3		3.3 ± 2.3	
Int-ENC-CFA				
Female	2.2 ± 0.7		2.1 ± 0.9	
Male	2.5 ± 1.1		2.7 ± 0.9	
Weight loss†				
Ext-ENC-CFA				
Female	3.5 ± 2.0		1.8 ± 2.1	
Male	5.7 ± 2.4	0.028	3.2 ± 2.7	<0.0001
Int-ENC-CFA				
Female	0.02 ± 1.9		0.9 ± 1.4	
Male	0.3 ± 3.2		0.9 ± 1.9	

*Percent affected.
†The average weight loss was calculated using the total change in grams for each animal from the baseline weight at injection to the peak of clinical disease.

the F_1 hybrid and 8% (37 of 494) of the F_2 intercross mice immunized with int-ENC-CFA developed clinical disease. In addition, ext-ENC-CFA immunized F_1 hybrid and F_2 intercross mice developed EAE earlier (15.4 ± 1.4 days *vs.* 23.6 ± 12.0 days; $P = 0.0021$ and 15.4 ± 4.4 *vs.* 21.1 ± 7.7 days; $P < 0.0001$, respectively) and more severe disease (3.8 ± 0.4 *vs.* 2.3 ± 0.8; $P < 0.0001$ and 3.1 ± 1.8 *vs.* 2.3 ± 0.9, $P = 0.0086$, respectively) compared to int-ENC-CFA immunized mice. Additionally, ext-ENC-CFA immunized mice exhibited significantly greater weight loss compared to int-ENC-CFA immunized animals (4.6 ± 2.4 *vs.* 0.1 ± 2.6 grams; $P < 0.0001$ and 2.5 ± 2.5 *vs.* 0.9 ± 1.6 grams; $P < 0.0001$, respectively).

Stratification of the populations by sex revealed that there was no significant difference in the clinical signs of disease between male and female mice immunized with ext-ENC-CFA with the exception of weight loss (Table 3). Both male F_1 hybrid and F_2 intercross ext-ENC-CFA immunized mice exhibited significantly greater weight loss compared to female mice (5.7 ± 2.4 *vs.* 3.5 ± 2.0 grams; $P = 0.028$ and 3.2 ± 2.7 *vs.* 1.8 ± 2.1 grams; $P < 0.0001$, respectively). Among the int-ENC-CFA immunized animals, the only significant sexual dimorphism observed was for disease incidence in the F_2 intercross population (24 of 233 female mice *vs.* 13 of 261 male mice; $P = 0.025$). These proportions (approximately 2:1) recapitulate our previous observations with this strain combination.[29]

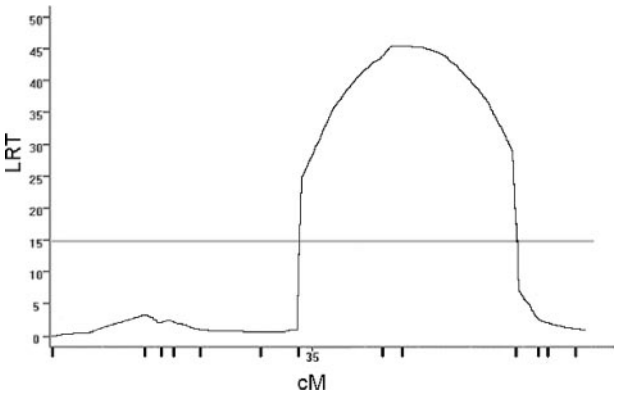

Figure 1. Genetic linkage on chromosome 7. CIM results for linkage of susceptibility to EAE (disease index) to chromosome 7 using the combined male and female F_2 intercross mice immunized with int-ENC-CFA emulsion. Permutation-derived significance cut-offs were based on 1000 permutations at $\alpha = 0.05$. Significance cutoffs for this trait are 14.8.

Given these results, it was of interest to map the QTL controlling differential susceptibility to EAE as a function of the MSCH-CFA emulsion. The F_2 intercross mice immunized with ext-ENC-CFA could not be used for this purpose due to complete penetrance and the lack of phenotypic variation among the mice. However, CIM based QTL analysis using disease index and severity were performed using 121 informative microsatellite markers on the 37 affected F_2 intercross mice immunized with int-ENC-CFA and 39 unaffected sex-matched littermates. The use of unaffected littermates allowed for better control of prenatal, perinatal, and postnatal environmental conditions, which may alter the immunological profile.[30,31]

Using this population, significant linkage of susceptibility to EAE (disease index) was detected to marker loci on chromosomes 7 (Figure 1) and 11 (Figure 2) (Table 4). Susceptibility induced with int-ENC-CFA was associated with B10.S alleles at both QTL. Suggestive linkage was also detected to *D18Mit24* on proximal chromosome 18 (LRT = 12.2; $P = 0.0022$) in a region where linkage has

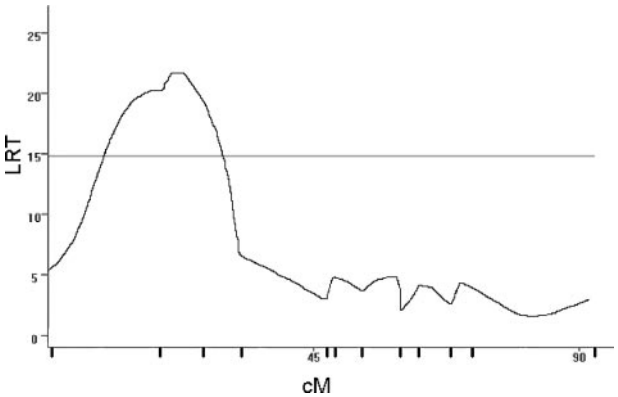

Figure 2. Genetic linkage on chromosome 11. CIM results for linkage of susceptibility to EAE (disease index) to chromosome 11 using the combined male and female F_2 intercross mice immunized with int-ENC-CFA emulsion. Permutation-derived significance cut-offs were based on 1000 permutations at $\alpha = 0.05$. Significance cutoffs for this trait are 14.8.

Table 4. Susceptibility to EAE Induced Using Int-ENC-CFA Emulsion is Linked to Marker Loci on Chromosomes 7 and 11*

Marker	Affected			Unaffected			χ^2	*P* value
	S	H	B	S	H	B		
D7Mit281								
Total	2	18	18	15	22	1	25.6	0.0001
Female	2	13	10	11	12	1	13.6	0.0011
Male	0	5	8	4	10	0	13.7	0.0011
D11Mit307								
Total	2	11	25	15	14	9	13.2	0.0013
Female	0	20	5	10	9	5	14.2	0.0008
Male	2	5	6	5	5	4	1.7	0.43

*Mice were classified as susceptible if they exhibited clinical signs for two or more consecutive days. *D7Mit281* and *D11Mit307* are the chromosome 7 and 11 markers exhibiting maximal linkage.

not been previously detected. Similar linkage results were obtained with disease severity for all three QTL (data not shown). Stratification by sex revealed that the suggestive linkage to proximal chromosome 18 (♀; LRT = 11.3; $P = 0.0036$ *vs.* ♂; LRT = 17.4; $P = 0.0002$), is primarily male-specific whereas linkage to the chromosome 11 (♀; LRT = 11.7; $P = 0.0029$ *vs.* ♂; LRT <6.0) is female specific. Previous analysis of the *eae6b* QTL, which co-localizes with this female-specific QTL in the present study, did not show female bias, and the SJL/J-derived allele was associated with higher severity.[17,29,32]

These linkage results indicate that the QTL on chromosomes 7 and 11 controlling susceptibility to EAE as a function of the particulate structure of the MSCH-CFA emulsions colocalized with *eae4* and *eae6b*.[17,32] Two obvious candidate genes for the QTL on chromosome 11 and *eae6b* are *IL12β* and the T-cell immunoglobulin and mucin domain containing gene-3 (*Timd3* now designated *Havcr2* for heptatitis A virus cellular receptor 2) located at ~20 cM on chromosome 11.[25] *Havcr2/Timd3* is a cell surface protein, found on Th_1-like T cells, that plays a role in regulating macrophage activation and has been implicated in EAE.[27] Therefore, we searched for *Havcr2/Timd3* locus-specific polymorphisms as well as structural polymorphisms in the gene product that may segregate with susceptibility and resistance to EAE. Two *Havcr2/Timd3* allele-specific genomic markers were developed and their segregation with susceptibility and resistance to EAE among inbred strains of mice examined (Table 5). No clear correlation was detected. Second, we sequenced the SJL/J and B10.S/SgMcdJ *Havcr2/Timd3* alleles to look for potential structural polymorphisms. Although there is one mutation between the nucleotide sequences of B10.S/SgMcdJ and SJL/J *Havcr2/Timd3* alleles, at the third base position of codon 76 (Thr); this change, A->G, is silent (Figure 3). Thus, there does not appear to be a structural polymorphism in the SJL/J and B10.S/SgMcdJ *Havcr2/Timd3* alleles that underlies linkage to *eae6b* or susceptibility to EAE, despite the fact that the *Havcr2/Timd3* locus itself is quite polymorphic. However, we did not do an exhaustive analysis to rule out the possible existence of polymorphic splice variants. With respect to *IL12β*, we previously sequenced the SJL/J and B10.S/SgMcdJ alleles and found no differences in the predicted amino acid sequences.[32]

To more fully explore *Havcr2/Timd3* and *IL12β* as candidates for *eae6b*, we examined the levels of expression of these two genes in SJL/J and B10.S/SgMcdJ mice. *Havcr2/Timd3* mRNA expression levels were assessed by real-time PCR using RNA from freshly isolated $CD4^+$ T cells, $CD4^+$ T cells stimulated *in vitro* with anti-CD3 and anti-CD28, or anti-CD3 and anti-CD28 plus IFNγ. There was no significant difference in the basal levels of expression of Havcr2/Timd3 by freshly isolated $CD4^+$ T cells between the two strains ($P = 0.10$) (Figure 4). Interestingly, stimulation *in vitro* resulted in a significant reduction in the levels of expression of *Havcr2/Timd3* in both

Table 5. *Havcr2/Timd3* Allotypes in EAE Susceptible and Resistant Strains of Mice

Strain	ASO*	SSLP†	RFLP‡	Haplotype§	*eae6b*¶
NOD/LtJ	*d*	197	+	a	Yes
Biozzi ABH	*c*	201	−	c	
B10.S/DvTe	*c*	199	−	b	Yes
SJL/J	*c*	201	−	c	
RIII.S/J	*c*	201	−	c	Yes
(B10.S/SgMcdJ, B10.A/SgSnJ)	*c*	199	−	b	
BALB/cByJ	*c*	201	−	c	Unknown
BALB/cJ	*c*	201	−	c	
PL/J	*c*	201	−	c	
SWR/J	*c*	191	−	e	
CBA/J	*c*	201	−	c	
C3H/HeJ	*c*	201	−	c	
DBA/2/J	*d*	205	+	d	
AKR/J	*d*	197	+	a	
C57BL/6J	*d*	205	+	d	

*Indicates the presence of either the DBA/2J (*d*) or BALB/c (*c*) *Havcr2* (formerly *Timd3*) allele as determined by using allele-specific oligonucleotide (ASO) primers. The presence of a signature codon in this fragment was confirmed by the detection of a *BstNI* site (at position 132 relative to the ATG) in the PCR product from each *d* strain.

†The fragment sizes of a polymorphic microsatellite marker within the *Havcr2/Timd3* locus is given in nucleotides, including the primer and flanking sequence.

‡Indicates the presence of a polymorphic *AflIII* restriction site at position 277 relative to the ATG.

§*Havcr2/Timd3* haplotypes based on the combined genotypes at the three polymorphic regions are indicated by a letter (a–e). The AKR/J subline sample (DNA from Jackson Laboratory) showed a different haplotype than the sequence in NCBI (AF450241).

¶Indicates if either significant or suggestive linkage of EAE to the *eae6b* region of chromosome 11 was observed in previous publications of the progeny of pairs indicated. For the RIII.S/J × B10.RIII/SnJ cross, the haplotype of B10.RIII/SnJ is inferred from the haplotype found in B10.S/DvTe and B10.A/SgSnJ.

```
C57BL/6          MFSGLTLNCVLLLLQLLLARSLENAYVFEVGKNAYLPCSYTLSTPGALVPMCWGKGFCPW
DBA/2            MFSGLTLNCVLLLLQLLLARSLENAYVFEVGKNAYLPCSYTLSTPGALVPMCWGKGFCPW
AKR              MFSGLTLNCVLLLLQLLLARSLEDGYKVEVGKNAYLPCSYTLPTSGTLVPMCWGKGFCPW
SJL/J            ----------LLLLQLLLARSLEDGYKVEVGKNAYLPCSYTLPTSGTLVPMCWGKGFCPW
B10.S/SgMcdJ     ----------LLLLQLLLARSLEDGYKVEVGKNAYLPCSYTLPTSGTLVPMCWGKGFCPW
                 ************************:.* .**************.*.*:**************

C57BL/6          SQCTNELLRTDERNVTYQKSSRYQLKGDLNKGDVSLIIKNVTLDDHGTYCCRIQFPGLMN
DBA/2            SQCTNELLRTDERNVTYQKSSRYQLKGDLNKGDVSLIIKNVTLDDHGTYCCRIQFPGLMN
AKR              SQCTNELLRTDERNVTYQKSSRYQLKGDLNKGDVSLIIKNVTLDDHGTYCCRIQFPGLMN
SJL/J            SQCTNELLRTDERNVTYQKSSRYQLKGDLNKGDVSLIIKNVTLDDHGTYCCRIQFPGLMN
B10.S/SgMcdJ     SQCTNELLRTDERNVTYQKSSRYQLKGDLNKGDVSLIIKNVTLDDHGTYCCRIQFPGLMN
                 ************************************************************

C57BL/6          DKKLELKLDIKAAKVTPAQTAHGDSTTASPRTLTTERNGSETQTLVTLHNNNGTKISTWA
DBA/2            DKKLELKLDIKAAKVTPAQTAHGDSTTASPRTLTTERNGSETQTLVTLHNNNGTKISTWA
AKR              DKKLELKLDIKAAKVTPAQTAHGDSTTASPRTLTTERNGSETQTLVTLHNNNGTKISTWA
SJL/J            DKKLELKLDIKAAKVTPAQTAHGDSTTASPRTLTTERNGSETQTLVTLHNNNGTKISTWA
B10.S/SgMcdJ     DKKLELKLDIKAAKVTPAQTAHGDSTTASPRTLTTERNGSETQTLVTLHNNNGTKISTWA
                 ************************************************************

C57BL/6          DEIKDSGETIRTAIHIGVGVSAGLTLALIIGVLILKWYSCKKKKLSSLSLITLANLPPGG
DBA/2            DEIKDSGETIRTAIHIGVGVSAGLTLALIIGVLILKWYSCKKKKLSSLSLITLANLPPGG
AKR              DEIKDSGETIRTAIHIGVGVSAGLTLALIIGVLILKWYSCKKKKLSSLSLITLANLPPGG
SJL/J            DEIKDSGETIRTAIHIGVGVSAGLTLALIIGVLILKWYSCKKKKLSSLSLITLANLPPGG
B10.S/SgMcdJ     DEIKDSGETIRTAIHIGVGVSAGLTLALIIGVLILKWYSCKKKKLSSLSLITLANLPPGG
                 ************************************************************

C57BL/6          LANAGAVRIRSEENIYTIEENVYEVENSNEYYCYVNSQQPS    XP_126117
DBA/2            LANAGAVRIRSEENIYTIEENVYEVENSNEYYCYVNSQQPS    AAL35776
AKR              LANAGAVRIRSEENIYTIEENVYEVENSNEYYCYVNSQQPS    AF450241
SJL/J            LANAGAVRIRSEENIYTIEENVYEVENSNEYYCYVNSQQPS
B10.S/SgMcdJ     LANAGAVRIRSEENIYTIEENVYEVENSNEYYCYVNSQQPS
```

Figure 3. cDNA sequences for *Havcr2/Timd3* alleles. SJL/J and B10.S/SgMcdJ were obtained using total RNA isolated from adult spleen. cDNAs were PCR-amplified using *Taq* polymerase and specific primer pairs flanking the mRNA coding region of the gene. The amplified fragments were TA-cloned and PCR-screened for inserts using gene-specific primers. At least three clones for each PCR fragment were sequenced from both insert termini. Although there is one mutation between the nucleotide sequences of B10.S/SgMcdJ and SJL/J *Havcr2/Timd3* alleles, at the third base position of codon 76 (Thr); this change, A->G, is silent.

strains. With unstimulated cells, there was, however, no significant difference in the level of *Havcr2/Timd3* expression between the two strains. IL12β expression levels were determined using unstimulated splenic RNA. Again, no significant difference in the level of expression of IL12β was seen between the two strains ($P = 0.24$) (Figure 5). These results are in agreement with earlier reports on IL12β secretion by SJL/J and B10.S/SgMcdJ macrophages[33]; however, a subsequent report shows that SJL/J mouse macrophages make more IL12 p40 when they are stimulated with lipopolysaccharide (LPS) compared with B10.S mouse macrophages.[34]

Discussion

In this study we used SJL/J, B10.S/SgMcdJ, (B10.S/SgMcdJ $\times$ SJL/J) F_1 hybrid, and (B10.S/SgMcdJ $\times$ SJL/J) F_2 intercross mice to demonstrate that MSCH-CFA emulsions consisting of particles where the *M. tuberculosis* and neuroantigens are localized on the phase external surfaces (ext-ENC-CFA) are significantly more encephalitogenic than MSCH-CFA emulsions in which the bacteria and neuroantigens are buried inside the water/oil vesicles (int-ENC-CFA). These results are consistent with those seen in inbred and congenic strains considered to be resistance to EAE immunized with ext-ENC-CFA emulsions generated by sonication.[35,36] Importantly, we see the same effects with or without the use of pertussis toxin as an ancillary adjuvant and when encephalitogenic peptides are used as the immunogen. Clearly, emulsification techniques and the resultant physical structure of the particles comprising the emulsions play a major role in their encephalitogenic activity and the genetic control of susceptibility and resistance to disease. In fact, the use of either ext-ENC-CFA and int-ENC-CFA emulsions accounts for greater phenotypic variation than the underlying genetic differences among susceptible and resistant inbred strains of mice. Additionally, our results support the hypothesis that the sexual dimorphism seen in EAE may be due in part to sex-specific QTL controlling re-

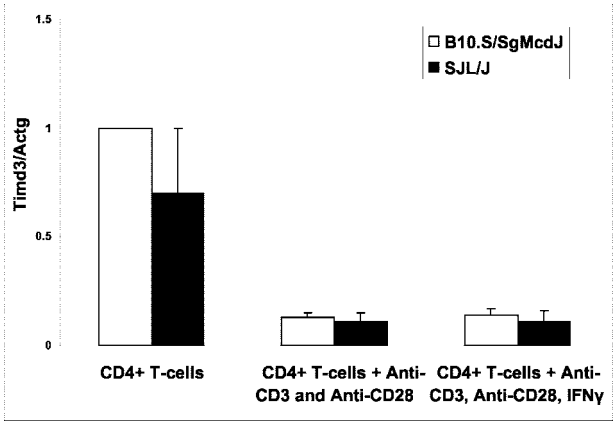

Figure 4. Havcr2/Timd3 expression by $CD4^+$ T cells. $CD4^+$ T cells were isolated from B10.S/SgMcdJ and SJL/J mice and *Havcr2/Timd3* expression levels assessed by TaqMan PCR using cDNA generated by reverse transcription of mRNA isolated from naïve cells and $CD4^+$ T cells stimulated with either anti-CD3 and anti-CD28 or anti-CD3 and anti-CD28 and IFNγ. Expression levels were normalized with respect to B10.S/SgMcdJ levels. Data are presented as the normalized means ± SD. No significant differences in *Havcr2/Timd3* expression were observed between B10.S/SgMcdJ and SJL/J under any of the conditions studies ($P > 0.05$).

sponsiveness to the adjuvants used to induce disease[16] and that the QTL identified on chromosomes 7 and 11 may function in this capacity.

Historically, significant species differences were observed in the adjuvanticity of oil-in-water and water-in-oil emulsions, and changes in both the structure and size of the particles comprising the emulsions have been shown to influence the type of immune response elicited.[35,37–44] In this regard, syringe-extrusion, high-speed homogenization and sonication lead to emulsions with markedly different physical structures. The particles generated by the later two techniques are much smaller than those generated by syringe-extrusion, and the *M. tuberculosis* and antigen are localized on the surface of the particles as compared to being within the particles.

Since dendritic cells (DCs) constitutively sample the local microenvironment via macropinocytosis and cell signaling using different receptors,[45] it is reasonable to assume that in emulsion where the *M. tuberculosis* and neuroantigens are localized on the phase surfaces effectively increases the antigen specific immunostimulatory activity of DCs compared to emulsions in which the bacteria and neuroantigens are buried inside the water/oil vesicles. DCs encountering the latter must first ingest the larger particles and then release the bacterial products and encephalitogens from within these particles before their activation and maturation, and the presentation of disease inducing epitopes to T cells[46,47]; dendritic cells that encounter ext-ENC-CFA emulsions do not.

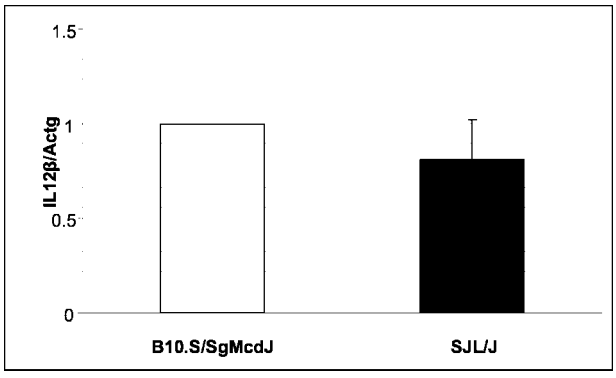

Figure 5. Il12β expression by SJL/J and B10.S/SgMcdJ splenic cells. IL12β expression was assessed by TaqMan PCR using cDNA generated by reverse transcription of mRNA isolated from individual, freshly excised spleens. Expression levels were normalized with respect to B10.S/SgMcdJ levels. Data are presented as the normalized means ± SD. Statistical significance was assessed using Student's *t*-test ($P = 0.24$).

Thus, the QTL controlling susceptibility to EAE induced with the less encephalitogenic int-ENC-CFA emulsions are likely to encode for genes that modify the ability of DCs to regulate or carry out one or more of these processes or activation states associated with these processes. *IL12β* and *Havcr2/Timd3*, located at ~19 and 20 cM on chromosome 11, respectively, are both associated with macrophage/T-cell activation and EAE severity[27,33] and are therefore attractive candidate genes the QTL identified on chromosome 11 in this study and *eae6b*. However, the lack of both structural and expression-level polymorphisms for *Havcr2/Timd3* between SJL/J and B10.S/SgMcdJ mice, combined with the discordance in *Havcr2/Timd3* allotypes among EAE-susceptible and -resistant inbred strains, suggests that it is excluded as a candidate. However, we did not do an exhaustive analysis to rule out the possible existence of polymorphic splice variants. Similarly, *IL12β* can be excluded as a candidate based on the same criteria.[32,33] Importantly, any candidate gene for the chromosome 11 QTL must take into account the sex specificity of the linkage observed.

The mechanisms underlying sex-specific QTL are unknown but may arise as a result of sex hormone regulation of the polymorphic genes underlying these QTL or interactions between mitochondrially or Y-chromosome-linked genes. The role of sex hormones in the sexual dimorphism observed in immune responsiveness as well as in immunopathologically based diseases has been well documented[48,49] as has the regulation of immunologically relevant genes such as cytokines.[50] With respect to potential interactions with mitochondrially linked genes, there is evidence to suggest that mutations in mitochondrially encoded genes contribute to an MS-like syndrome, Leber's hereditary optic neuropathology, which occurs primarily in women.[51] Such mutations, however, do not appear to be a risk factor for MS *per se*.[52] Additionally, a number of immunologically relevant genes are known to be on the X-chromosome (http://www.informatics.jax.org/searches/linkmap.cgi).

Although somewhat speculative, it is worth noting several candidate genes residing within the chromosome 7 QTL interval. *Pgia3* (proteoglycan induced arthritis-3) and *Cia7* (collagen-induced arthritis QTL-7) at 42.6 cM and 50.0 cM, respectively, are of interest in light of the recent validation of the shared autoimmune disease susceptibility gene hypothesis.[13–15] Additionally, *Tria4*, a QTL controlling T-cell receptor-induced activation, resides within the interval as do a number of immunologically relevant candidate genes. These include *Art2a* and *Art2b* (ADP-ribosyltransferase 2a and 2b), *Art1* and *Art4* (ADP-

ribosyltransferase 1 and 2), *Cd19*, *Ly14*, *Itgal*, *Mph1* (macrophage antigen-1), and *Spn* (sialophorin or CD43) (www.informatics.jax.org/searches/linkmap.cgi). Two additional candidate genes of particular relevance to our hypothesis that the QTL identified in this study may play a role in the efficiency of processing and presenting antigens to T cells as a function of the emulsions particulate microstructure are *Aqp8* (aquaphorin 8) at 61.0 cM and *Psma1* (proteosome subunit, α type 1 or macropain) at 53.0 cM especially given their respective roles in antigen uptake, concentration, processing, and presentation to T cells.[53–56] Clearly, a structural or expression-level polymorphism that affects the ability of aquaporins to facilitate this process, or degrade the concentrated macrosolutes (*Psma1*), could lead to significant quantitative differences in disease. Interestingly, *Psma1* maps to 11p15.1 in the human, which is in an MS susceptibility region at 11p15 identified by the Multiple Sclerosis Genetics Group in 1996.[57]

Several EAE genetics studies in the mouse have been completed during the past few years.[16,17,32,58–64] These studies were carried out with little regard for the importance that the microstructure of the particles comprising the CFA emulsions play in disease susceptibility. In light of our results and those reported previously,[35,36] this variable may be singularly responsible for failing to identify a more uniform, robust set of susceptibility loci across these studies. However, the results of this study and those of Baker et al[59] do exhibit a significant degree of concordance in that the most significant linkages observed in both studies were to chromosomes 7 (*eae4*), 11 (*eae6b*), and 18 and int-ENC-CFA emulsions were used to immunize the mapping populations.

Our results raise the intriguing possibility that similar genetically controlled mechanisms may play a role in the gene-environment interactions underlying the disease heterogeneity seen in MS.[65] It is highly likely that some of the genes controlling susceptibility to MS control the host's response to the microstructure-based adjuvant effects of the infectious or environmental agents associated with MS rather than their infectivity, latency, or the ability of the anticipatory immune system to mount a pathogenic T- or B-cell immune response via molecular mimicry. This appears to be the case with respect to particulate pollution.[66] For example, the IgE and IgG3a antigen-independent, adjuvant-enhancing effects of physically and chemically defined particles is also genetically controlled. Clearly, a better understanding of the genes, their products, and the pathways controlling responsiveness to immunogens and infectious agents solely as a function of the physicality of their microstructure may lead to an improved understanding of how specific environmental and genetic factors, and their interactions, are involved in initiating and propagating immunopathologically based diseases.

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