Immunopathology and Infectious Diseases

Gender, Age, and Season at Immunization Uniquely Influence the Genetic Control of Susceptibility to Histopathological Lesions and Clinical Signs of Experimental Allergic Encephalomyelitis

Implications for the Genetics of Multiple Sclerosis

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Multiple sclerosis (MS), the principal inflammatory demyelinating disease of the central nervous system (CNS), is believed to have an immunopathological etiology arising from gene-environment interactions. In this study, we examined the effect of sex, age, and season at immunization on the susceptibility of (B10.S × SJL/J) F₂ intercross mice to experimental allergic encephalomyelitis (EAE), the foremost animal model of MS. Results of logistic regression analyses suggest that female mice were more likely to exhibit CNS lesions than male mice [odds ratio (OR) = 2.28 for brain lesions; OR = 2.37 for spinal cord (SC) lesions]. Although statistically significant associations were seen between brain and SC lesions and age at the time of injection or month of injection when examined separately; these associations disappeared when controlling for sex in multiple logistic regression analyses. These results suggest that the sex of the mouse is more important in influencing the development of brain and SC lesions than was either age or month of immunization. When examining clinical disease as the endpoint, the OR for the age at immunization is 1.04, indicating that the odds of being affected increase by 4% for each increasing week of age. When controlled for age, the OR for injection in the summer months (July through September) is 1.90, suggesting that the odds of being clinically affected are 90% greater for F₂ intercross animals injected in the summer

compared to those injected in the winter to spring months (February through May). In contrast to CNS lesions, the age and season at immunization significantly and independently influenced susceptibility to clinical EAE and did so equally in both males and females. Linkage analysis to *eae5*, the H2-linked locus controlling susceptibility to clinical disease, was performed using 6- to 12- and >12-week-old cohorts as well as summer and winter/spring cohorts of F₂ mice. Significant linkage of clinical EAE to *eae5* was observed with the 6- to 12-week-old and summer populations. In contrast, linkage of clinical EAE to *eae5* was not detected with the >12-week-old and winter/spring populations. These results indicate that age and seasonal effects are capable of overriding *eae5*-dependent genetic control of susceptibility to clinical EAE and have significant implications for the genetics of MS. (*Am J Pathol* 2004, 165:1593–1602)

Multiple sclerosis (MS) is the major inflammatory demyelinating disease of the central nervous system (CNS) in humans.^{1–3} MS presents primarily in young adults and exists in several subtypes ranging from malignant MS, a rapidly progressive and fatal form of the disease, to a benign MS that in some patients is only detected on autopsy or after magnetic resonance imaging.^{1,4} The etiology of MS is unknown but is believed to have an immunopathological basis arising in genetically predisposed individuals as a consequence of an environment insult(s) followed by a protracted latent period^{5–10} with a mean age

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of onset of 27 years.⁵ The etiological role of a variety of environmental factors is supported by epidemiological studies and include sociocultural (social class, educational level, health level, migration and multiple moves, occupation, and exposure to toxins), biological (age, infectious agents, vaccination, diet and daily nutrition, pets, trauma and stress, pregnancies, and surgical procedures), and physical (season, latitude, altitude, climate, water and soil composition, and chemical and physical agents) factors.¹¹ Moreover, it is possible that the contribution of environmental factors may depend on interaction with host intrinsic factors such as age and sex.¹²

Experimental allergic encephalomyelitis (EAE) is the principal animal model of MS and is elicited by immunization with CNS antigens and the appropriate adjuvants.^{13,14} Like MS, EAE is elicited in genetically susceptible hosts that exhibit histopathological lesions and a spectrum of clinical disease subtypes that are genetically controlled and recapitulate those observed in MS,¹⁵ including optic neuritis.¹⁶ Importantly, EAE is also subject to environmental influences.¹⁷ For example, modifying the disease induction protocol to include pertussis toxin as an ancillary adjuvant overrides many of the genetic checkpoints identified when complete Freund's adjuvant (CFA) is used as the sole adjuvant.¹⁸ The use of pertussis toxin results in less severe disease in female mice compared to female mice immunized without pertussis toxin. In this same study, immunization with and without pertussis toxin also significantly impacted a number of the sexually dimorphic susceptibility loci. Another extrinsic variable, the physical structure of the particles comprising the neuroantigen-CFA emulsions, was also shown to impact both the sexual dimorphism in EAE and the genetic control of susceptibility and resistance.¹⁹

Given that environmental factors influence susceptibility to MS and EAE, we examined the role of sex, age, and season at immunization on susceptibility to histopathological and clinical EAE in the context of genetic heterogeneity. We report that sex and the age and season at immunization significantly and independently influence susceptibility to autoimmune disease of the CNS; the impact of age and season extend to genetic linkage to *eae5*, the primary locus controlling susceptibility to clinical EAE linked to the murine major histocompatibility complex (*H2*).^{20,21} Importantly, our results indicate that there are environmentally sensitive genes linked to the major histocompatibility complex that control susceptibility to the clinical signs associated with autoimmune disease of the CNS. The existence of such loci may explain why fine mapping studies have failed to definitively identify the *HLA*-linked MS susceptibility gene(s) despite the fact that linkage to *HLA* is the single most consistent finding in MS genetic studies.¹⁰ They also have the potential to represent examples of Simpson's paradox: a statistical phenomenon in which the effects at a locus can be masked, enhanced, or even reversed by gene-gene or gene-environment interactions that are not detected and accounted for.²²⁻²⁵

Materials and Methods

Animals

Male and female SJL/J and B10.S mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The 1673 mice used in this study comprise 681 (B10.S $\times$ SJL/J) F₂ mice used in our earlier studies^{20,21} and an additional 992 multidirectional F₂ mice generated at the University of Illinois at Urbana-Champaign. Male and female (B10.S $\times$ SJL/J) and (SJL/J $\times$ B10.S) F1 hybrid mice were generated and (B10.S $\times$ SJL/J) $\times$ (B10.S $\times$ SJL/J); (B10.S $\times$ SJL/J) $\times$ (SJL/J $\times$ B10.S); (SJL/J $\times$ B10.S) $\times$ (B10.S $\times$ SJL/J); and (SJL/J $\times$ B10.S) $\times$ (SJL/J $\times$ B10.S) F₂ mice were produced by intercrossing F1 hybrid animals. Multidirectional intercross mice were added to the sample set to maximize genetic heterogeneity to more closely model the genetic architecture underlying MS. 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. Animals were maintained under standard environmental conditions including controlled temperature, humidity and 12:12 hour light:dark cycle. Additionally, the infectious disease status of the colony was monitored serologically using a standard sentinel program. No change in the serological profile of the animals was observed throughout the course of the experiments.

Preparation of Mouse Spinal Cord (SC) Homogenate (MSCH)-CFA Emulsions

MSCH was generated using retired breeder SJL/J mice purchased from either the Jackson Laboratory or Charles River Laboratory, Wilmington, MA. SCs were extracted by insufflation, mixed with an equal volume of distilled H₂O, homogenized in a Pyrex tissue homogenizer, and filtered through nylon mesh. The MSCH was lyophilized and resuspended at 50 mg/ml in phosphate-buffered saline (PBS) and 1-ml aliquots were stored at -70°C until used. MSCH-CFA emulsions were prepared by syringe extrusion 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 *Mycobacterium 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 in the oil phase. Subsequently, the plungers were depressed alternately until a nonwater surface separating emulsion was achieved.

Induction and Evaluation of EAE

Mice were immunized for the induction of EAE as previously described.²⁰ 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 of dry weight SJL/J MSCH and 30.0 μ g of *Mycobacterium tuberculosis* H37Ra. Different preparations of SJL MSCH, incomplete Freund's adjuvant, and *M. tuberculosis* H37Ra were used to immunize the two cohorts of F2 mice described in this study; however, within each cohort the preparations were uniform. Mice were scored as affected and unaffected starting at day 10 after injection through day 60. Animals were scored daily between 12:00 to 18:00 hours. All animals were considered affected that exhibited any clinical signs greater or equal to a flaccid tail and/or hind leg weakness for 2 or more consecutive days.

Brains and SCs were dissected from calvaria and vertebral columns, respectively, and fixed by immersion in 10% phosphate-buffered formalin (pH 7.2). After adequate fixation, they were trimmed and representative transverse section-embedded in paraffin, sectioned at 3 μ m, and mounted on glass slides. Sections were stained with hematoxylin and eosin for routine evaluation and Luxol fast blue-periodic acid-Schiff reagent for demyelination. Representative areas of the brain and SC, including brain stem, cerebrum, cerebellum, and the cervical, thoracic, and lumbar segments of the SCs, were selected for histopathological evaluation based on previous studies.²¹ Histopathological lesions in EAE are primarily characterized by inflammation and demyelination.²¹ Lesions in the brain and SC were evaluated separately and assigned a numerical score based on a subjective scale ranging from 0 to 5. A score of 0 indicates no lesions; 1 indicates minimal; 2, mild; 3, moderate; 4, marked; and 5, severe lesions. The following components of the lesions as demonstrated in Figure 1 were assessed: 1) severity and extent of the lesion; 2) extent and degree of myelin loss and tissue injury (swollen axon sheaths, swollen axons, and reactive gliosis); 3) severity of the acute inflammatory response (predominantly neutrophils); and 4) severity of the chronic inflammatory response (lymphocytes/macrophages). Animals exhibiting any of the lesion components were scored as affected in this study.

Statistical Analysis

A bivariate logistic regression analysis was used to examine the relationship between histological evidence of, or clinical susceptibility to, EAE and each of the independent variables of interest, including month at injection, sex and age at the time of injection, with age (in weeks) being considered as a continuous variable. This was followed by multiple logistic regression analyses to examine the effects of these variables on disease susceptibility while controlling for each of the other selected variables. All analyses were performed using SAS System for Windows, version 8.1 (SAS Institute Inc., Cary, NC).

Linkage Analysis

Informative chromosome 17 microsatellite primers were either purchased from Research Genetics (Huntsville, AL) or synthesized according to sequences obtained through Mouse Genome Informatics. Polymerase chain reaction parameters for microsatellite typing were previously described.²⁰ Microsatellite size variants were resolved by electrophoresis on large format denaturing polyacrylamide gels and visualized by autoradiography on Kodak film (Eastman-Kodak, Rochester, NY). Linkage of EAE to chromosome 17 markers was assessed using a χ^2 test for each marker locus in 2×3 contingency tables.

Results

A total of 1673 mice were injected throughout the months of February, April, May, July, August, and September. The mice ranged in age from 6 to 44 weeks at the time of injection, with the average age being 19.0 ± 10.2 weeks. Male and female mice were represented in approximately equal numbers (Table 1). Of these mice, 1163 were examined for the presence of inflammatory infiltrates in the CNS, both in the brain and in the SC.

The results of the logistic regression analyses for brain lesions are presented in Table 2. Age at the time of injection and sex, were significantly associated with inflammation in the brain (Table 2). The odds ratio (OR) for age at the time of injection was 1.03, indicating that the odds of developing inflammatory infiltrates within the brain increase by 3% for each increasing week of age at the time of injection. Female F₂ mice were highly significantly more likely to show this parameter than male F₂ mice (OR, 2.3). In addition, animals injected during April were more likely to exhibit brain inflammation than those injected during February (OR = 3.06). The ORs for injection during months other than April were not elevated. Because the age distribution of injected mice differed by month, a multiple logistic regression was performed to examine the effect of all variables simultaneously. The OR for female mice was unchanged (Table 3) but the significance of the other factors was greatly reduced. Mice injected in August were less likely to develop brain lesions (OR = 0.55) than mice injected in February; ORs for injection during all other months were not significantly different from 1.00. These results suggest that sex is important in influencing the development of brain lesions; however, after controlling for the effect of sex, age, and month of injection are not associated with brain inflammation. Similar results were found for SC lesions, which were much more likely to be seen in female mice than male mice; the multiple logistic analyses indicate that the apparent separate influences of age and month of injection are reduced to nil (Tables 4 and 5).

The results of logistic regression analyses on susceptibility to clinical disease are presented in Table 6. Both age at the time of injection and the month of injection were significantly related to susceptibility, but there was no significant association with the sex of the mice. The OR for age at the time of injection was 1.04, indicating

Figure 1. Characteristic histopathological lesions in murine EAE. **A:** Normal SC white matter under the pia mater. **B:** Myelin stained (light blue) in normal SC. **C:** Pia mater and subpial white matter is infiltrated (perivascular pattern) by acute inflammatory cells consisting predominately of neutrophils and lesser numbers of chronic inflammatory cells (lymphocytes/macrophages). There is extensive destruction of the subpial white matter with swelling and fragmentation of axon sheaths (**arrows**) and reactive gliosis. **D:** There is extensive demyelination (loss of light blue staining) in the subpial white matter. **E:** Normal brain white matter (cerebrum, external capsule). **F:** Myelin stained (light blue) in normal external capsule of the cerebrum. **G:** The perivascular space in the external capsule is infiltrated by lymphocytes (**arrowhead**). **H:** Demyelination is absent or minimal in areas of chronic inflammation. **Left**, H&E stain; **right**, Luxol fast blue-periodic acid-Schiff stain. Scale bar, 50 μ m.

Table 1. Distribution of Gender and Month at Injection of F₂ Intercross Mice Studied for CNS Lesions and Clinical Signs of EAE

Independent variable	Lesions (%)	Clinical (%)
Sex		
Male	525 (45.1)	841 (50.3)
Female	638 (54.9)	832 (49.7)
Month at injection		
February	114 (9.8)	139 (8.3)
April	120 (10.3)	228 (13.6)
May	—	60 (3.6)
July	263 (22.6)	409 (24.4)
August	282 (24.2)	430 (25.7)
September	384 (33.0)	407 (24.3)

that the odds of being affected increase by 4% for each increasing week of age at the time of injection. The month of injection was also associated with being affected, with those injected in April, May, and August more likely to be affected than mice injected in February. Mice injected in July or September were not more likely to be affected than those injected in February.

The results of the multiple logistic regression analyses are presented in Table 7. Because sex was not associated with being affected in the univariate analyses, nor did it appear to confound any of the relationships between other independent variables in the multiple logistic

Table 2. Bivariate Associations between Age, Sex, or Month at Injection and Brain Lesions

Independent variable	Odds ratio	95% CI*	P value
Age (in weeks)	1.03	1.0, 1.05	<0.001
Sex			
Male	1.00 [†]		
Female	2.28	1.78, 2.92	<0.001
Month at injection			
February	1.00 [†]		
April	3.06	1.63, 5.76	<0.001
July	0.87	0.55, 1.37	0.54
August	0.59	0.38, 0.92	0.02
September	1.46	0.94, 2.30	0.10

*Confidence interval.
[†]Reference variable.

Table 3. Multiple Logistic Model Coefficients Table for Brain Lesions

Independent variable	Odds ratio	95% CI*	P value
Age (in weeks)	1.01	0.98, 1.05	0.55
Sex			
Male	1.00 [†]		
Female	2.42	0.88, 3.13	<0.001
Month at injection			
February	1.00 [†]		
April	2.36	0.95, 5.84	0.06
July	0.93	0.55, 1.58	0.78
August	0.55	0.34, 0.88	0.01
September	1.58	0.95, 2.64	0.08

*Confidence interval.
[†]Reference variable.

Table 4. Bivariate Associations between Age, Sex, and Month at Injection and SC Lesions

Independent variable	Odds ratio	95% CI*	P value
Age (in weeks)	1.02	1.01, 1.04	<0.01
Sex			
Male	1.00 [†]		
Female	2.37	1.85, 3.04	<0.001
Month at injection			
February	1.00 [†]		
April	2.39	1.34, 4.27	<0.01
July	1.03	0.65, 1.61	0.92
August	1.20	0.76, 1.88	0.43
September	1.22	0.79, 1.88	0.37

*Confidence interval.
[†]Reference variable.

regression analyses, it was deleted from subsequent models. When examined together with month of injection, the affect of age at the time of injection was similar to that which was seen in the univariate analysis, with an OR of 1.04 for each 1-week increase in age. The pattern of month at injection, however, was somewhat altered in model 1, as compared to that seen in the bivariate analyses. Controlling for age at the time of injection, those mice injected in July, August, and September were significantly more likely to become affected than those injected in February (OR = 1.75, 2.13, and 1.64, respec-

Table 5. Multiple Logistic Model Coefficients Table for SC Lesions

Variable	Odds ratio	95% CI*	P value
Age (in weeks)	1.01	0.98, 1.04	0.59
Sex			
Male	1.00 [†]		
Female	2.34	1.82, 3.00	<0.001
Month at injection:			
February	1.00 [†]		
April	1.88	0.80, 4.22	0.15
July	1.09	0.65, 1.84	0.74
August	1.15	0.71, 1.84	0.57
September	1.30	0.79, 2.14	0.30

*Confidence interval.
[†]Reference variable.

Table 6. Bivariate Associations between Age, Month at Injection, or Sex and Susceptibility to Clinical Disease

Independent variable	Odds ratio	95% CI*	P value
Age (in weeks)	1.04	1.02, 1.05	<0.001
Sex			
Male	1.00 [†]		
Female	1.18	0.97, 1.43	0.11
Month at injection			
February	1.00 [†]		
April	3.32	2.11, 5.24	<0.001
May	1.92	1.02, 3.60	0.04
July	1.20	0.81, 1.76	0.37
August	1.66	1.13, 2.45	0.01
September	1.09	0.74, 1.61	0.65

*Confidence interval.
[†]Reference variable.

Table 7. Age and Month at Injection Effects on Susceptibility to Clinical Disease

Independent variable	Odds ratio	95% CI*	P value
Model 1			
Age	1.04	1.02, 1.07	<0.01
Month at injection			
February	1.00 [†]		
April	1.65	0.88, 3.11	0.12
May	0.93	0.43, 2.02	0.85
July	1.75	1.11, 2.75	0.02
August	2.13	1.40, 3.24	<0.001
September	1.64	1.03, 2.60	0.04
Model 2			
Age	1.06	1.04, 1.08	<0.001
Season at injection			
Winter/Spring	1.00 [†]		
Summer	1.90	1.27, 2.85	<0.01

*Confidence interval.

[†]Reference variable.

tively). Those mice injected in April or May were not more likely to be affected than those injected in February. The months at injection were then collapsed into seasons, with injection in the summer months (July, August, and September) being compared to injection in the winter/spring (February, April, May), with the results presented as model 2. As in model 1, the age in weeks was associated with an increase in the odds of being affected. When controlling for age, the OR for injection in the summer months was 1.90, suggesting that the odds of being affected are 90% higher for those injected in the summer, compared to those injected in the winter/spring.

These results suggested that the age and season at immunization may influence genetic checkpoints controlling susceptibility to clinical EAE.^{18,19} To test this hypothesis, we selected age and season disparate (B10.S × SJL/J) F₂ mapping populations to assess linkage to *eae5*, the principal locus controlling susceptibility to clinical disease linked to *H2*.^{20,21} The animals were arbitrarily divided into season-matched cohorts ranging in age from 6 to 12 weeks and >12 weeks to examine the effects of age at immunization. To examine the effects of season at

immunization, the animals were stratified into age-matched winter/spring and summer season cohorts according to model 2 above. Both data sets were analyzed for linkage to chromosome 17 marker loci.

The linkage results obtained with age and season disparate animals are presented in Table 8. Significant linkage of clinical disease to *eae5* was detected with maximal linkage to *D17Mit176* at 22.5 cM ($\chi^2 = 22.2$, $P = 1.5E-5$). When the 6- to 12-week-old population was used, significant linkage to *eae5* was also detected with maximal linkage to *D17Mit176* at 22.5 cM ($\chi^2 = 18.7$, $P = 8.7E-5$) (Table 9). In contrast, no linkage was seen to marker loci when the analysis was performed using the >12-week-old cohort. Similarly, no significant linkage to chromosome 17 marker loci was detected when the analysis was done using the winter/spring cohort (Table 10). However, significant linkage of clinical disease to chromosome 17 marker loci was detected with the summer cohort; maximal linkage being to *D17Mit176* at 22.5 cM ($\chi^2 = 22.2$, $P = 1.5E-5$). A comparison of the significance of linkage to *D17Mit176* of the 6- to 12-week-old and summer cohorts with that obtained using all of the animals ($\chi^2 = 22.2$, $P = 1.5E-5$) indicates that little linkage is being contributed by the corresponding cohorts. Similar effects were seen for all chromosome 17 marker loci.

Discussion

In this study, we used a large F₂ intercross population generated using the susceptible SJL/J and resistant B10.S inbred strains of mice to examine the effects of sex, age, and season at immunization on susceptibility and resistance to histopathological and clinical EAE. Multidirectional intercross mice were used to maximize genetic heterogeneity to more closely model gene-environment interactions in MS. In this context, logistic regression analyses indicate that the sex of the mouse is significantly more important in influencing the development of lesions in the brain and SC than is either age or season at immunization. In contrast, our results clearly

Table 8. Linkage of Clinical Disease to *eae5* in Age and Season Disparate (B10.S/DvTe × SJL/J) F₂ Intercross Mice

Marker	cM	Affected			Unaffected			χ^2	P
		B*	H	S	B	H	S		
<i>D17Mit97</i>	7.1	72	182	87	102	206	75	5.1	0.08
<i>H2-K</i>	18.44								
<i>H2-D</i>	19.0								
<i>H2-M1</i>	20.23								
<i>D17Mit105</i>	21.95	51	203	90	104	210	85	14.3	0.0008
<i>D17Mit176</i>	22.5	52	203	90	112	185	79	22.2	1.5E-5
<i>D17Mit51</i>	22.9	54	201	90	110	209	81	15.8	0.0003
<i>D17Mit200</i>	24.2	58	176	85	114	198	76	13.4	0.001
<i>D17Mit115</i>	24.2	56	195	94	104	176	81	16.0	0.0003
<i>D17Mit70</i>	32.7	54	183	92	106	184	74	17.1	0.0001
<i>D17Mit20</i>	34.2	64	191	86	108	193	82	9.0	0.01
<i>D17Mit89</i>	36.0	69	174	83	107	199	85	4.0	0.13
<i>D17Mit3</i>	41.5	75	167	83	107	205	89	1.8	0.41
<i>D17Mit72</i>	47.4	86	172	87	125	206	75	6.2	0.04
<i>D17Mit221</i>	56.7	88	160	87	100	215	87	2.8	0.25

*B, H, and S represent the B10.S, heterozygote, and SJL/J genotypes at each locus, respectively.

Table 9. Age at Immunization Effect on Linkage of Clinical Disease to *eae5* in (B10.S/DvTe × SJL/J) F₂ Intercross Mice

Marker	cM	6 to 12 weeks								>12 weeks							
		Affected			Unaffected			χ^2	<i>P</i>	Affected			Unaffected			χ^2	<i>P</i>
		B*	H	S	B	H	S			B	H	S	B	H	S		
<i>D17Mit97</i>	7.1	30	92	47	46	92	30	7.1	0.03	36	71	35	38	72	26	1.3	0.53
<i>H2-K</i>	18.44																
<i>H2-D</i>	19.0																
<i>H2-M1</i>	20.23																
<i>D17Mit105</i>	21.95	21	99	50	49	98	35	13.5	0.001	25	84	34	34	67	32	3.0	0.22
<i>D17Mit176</i>	22.5	22	100	49	53	79	32	18.7	8.7E-5	25	84	34	38	65	29	5.1	0.08
<i>D17Mit51</i>	22.9	22	99	50	52	96	32	15.9	0.0003	27	83	33	38	67	30	3.5	0.18
<i>D17Mit200</i>	24.2	26	91	46	50	90	29	11.3	0.003	28	71	34	43	64	30	3.7	0.15
<i>D17Mit115</i>	24.2	23	96	52	47	84	35	12.3	0.002	28	81	34	36	52	30	5.2	0.07
<i>D17Mit70</i>	32.7	21	91	52	47	82	30	16.2	0.0003	28	73	34	40	63	31	3.0	0.22
<i>D17Mit20</i>	34.2	26	96	49	48	92	36	8.5	0.01	32	75	32	42	66	35	2.0	0.37
<i>D17Mit89</i>	36.0	29	87	48	46	93	37	5.1	0.08	33	71	29	42	60	32	2.2	0.34
<i>D17Mit3</i>	41.5	32	85	49	47	94	40	3.6	0.17	38	70	30	40	70	32	0.1	0.97
<i>D17Mit72</i>	47.4	39	85	47	55	90	35	4.4	0.11	38	70	35	44	72	28	1.2	0.53
<i>D17Mit221</i>	56.7	40	79	45	43	101	37	2.8	0.25	39	66	36	32	75	36	1.3	0.53

*B, H, and S represent the B10.S, heterozygote, and SJL/J genotypes at each locus, respectively.

demonstrate that there is a significant effect of both age and season at immunization on susceptibility to clinical EAE, and that both effects are independent of sex in that they equally influence males and females.

Age-dependent effects on susceptibility to EAE have been reported for both active and passive disease in inbred SJL/J mice. SJL/J male mice have a developmental delay in EAE-susceptibility not seen in female mice. This age-associated sexual dimorphism has been observed in multiple settings in SJL/J mice^{26–31} including a myelin basic protein adoptive transfer model.³² However, to our knowledge this is the first study examining the effects of age on susceptibility to clinical EAE in the context of genetic heterogeneity. For the effect of age at injection on clinical disease we obtained an OR of 1.04, indicating that the odds of exhibiting clinical signs increase by 4% for each increasing week of age. These observations have relevance to MS, which also shows evidence for age-related changes in disease susceptibility and subtype.^{5,33,34}

The seasonal effect identified in this study, when controlling for age, indicates that the odds of exhibiting clinical signs of EAE are 90% higher for mice injected in the summer, compared to those injected in the winter/spring. To our knowledge this is the first documentation of this effect in EAE, despite the fact that it is a well-known phenomenon among researchers in the field. The seasonal effect not only impacts the development of clinical signs in actively induced disease but also influences passively induced disease: greater numbers of encephalitogenic T cells are often required to elicit similar levels of clinical disease at different times of the year (multiple personal communications).

The results of these studies indicate that the sex of the mouse more significantly influences susceptibility to histopathology than does age and season at immunization. In contrast, age and season at immunization have a greater impact on the expression of clinical EAE. Thus, age and season at immunization likely influence the mechanisms underlying the manifestation of clinical

Table 10. Season at Immunization Effect on Linkage of Clinical Disease to *eae5* in (B10.S/DvTe × SJL/J) F₂ Intercross Mice

Marker	cM	Winter/Spring								Summer							
		Affected			Unaffected			χ^2	<i>P</i>	Affected			Unaffected			χ^2	<i>P</i>
		B*	H	S	B	H	S			B	H	S	B	H	S		
<i>D17Mit97</i>	7.1	23	53	21	31	59	25	0.3	0.84	49	129	66	71	147	50	6.3	0.04
<i>H2-K</i>	18.44																
<i>H2-D</i>	19.0																
<i>H2-M1</i>	20.23																
<i>D17Mit105</i>	21.95	16	60	22	26	63	28	1.5	0.47	35	143	68	78	147	57	15.0	0.0006
<i>D17Mit176</i>	22.5	16	59	23	27	56	28	2.6	0.28	36	144	67	85	129	51	22.2	1.5E-5
<i>D17Mit51</i>	22.9	18	59	21	27	62	28	1.2	0.55	36	142	69	83	147	53	18.4	0.0001
<i>D17Mit200</i>	24.2	18	49	21	32	56	28	1.6	0.46	40	127	64	82	142	48	14.3	0.0008
<i>D17Mit115</i>	24.2	19	56	23	29	53	26	1.9	0.39	37	139	71	75	123	55	15.8	0.0004
<i>D17Mit70</i>	32.7	20	54	23	30	51	24	1.8	0.41	34	129	69	76	133	50	17.7	0.0001
<i>D17Mit20</i>	34.2	22	54	22	28	48	25	1.2	0.54	42	137	64	80	145	57	9.6	0.008
<i>D17Mit89</i>	36.0	23	48	21	28	58	27	0.03	0.98	46	126	62	79	141	58	6.0	0.05
<i>D17Mit3</i>	41.5	23	47	18	30	56	26	0.3	0.87	52	120	65	77	149	63	2.9	0.23
<i>D17Mit72</i>	47.4	27	52	19	39	63	17	1.3	0.51	59	120	68	86	143	58	4.9	0.09
<i>D17Mit221</i>	56.7	30	40	27	25	64	27	4.3	0.12	58	120	60	75	151	60	1.3	0.51

*B, H, and S represent the B10.S, heterozygote, and SJL/J genotypes at each locus, respectively.

signs subsequent to CNS infiltration and lesion formation. This would predict that the loci governing clinical EAE might be sensitive to this influence, and in fact, *eeae5* is one such locus, with no known linkage to any histopathological parameter.²¹ This interpretation is also supported by the fact that *eeae5* is not sexually dimorphic but is age and seasonally dimorphic. Importantly, among the loci influencing clinical EAE and its subphenotypes in mice with underlying histopathological disease, a number are nonsexually dimorphic but are age and/or seasonally dimorphic (unpublished data).

The confirmation of seasonal variation in susceptibility to clinical EAE in the context of genetic heterogeneity is of particular significance with respect to EAE as a model for MS. Seasonal variation in the prevalence of clinical exacerbations in MS is well documented.^{35–40} Additionally, seasonal variation in the clinical onset of monosymptomatic optic neuritis, considered a *forme fruste* of MS,^{41–50} has been reported.⁵¹ A recent meta-analysis using data from nine reports on monosymptomatic optic neuritis, six reports on MS onset, and nine reports on MS exacerbations, indicates that the onset of monosymptomatic optic neuritis and MS as well as MS exacerbations in the Northern hemisphere present a similar pattern with highest frequencies in spring/summer and lowest in winter.⁵²

The mechanism underlying the seasonal effect on susceptibility to clinical EAE is of particular interest. Biological rhythms have been observed in practically all groups of laboratory mammals and at every level of behavioral and physiological organization.^{53,54} Such rhythms are classified according to their period as ultradian (<24 hours), circadian (~24 hours), infradian (>24 hours), and seasonal or circannual (~1 year).⁵³ Although many biological rhythms are related to external environmental cycles (exogenous rhythms), such as the daily light-dark cycle or seasonal changes in day length and temperature, many persist in the laboratory even under highly standardized conditions, and are therefore considered endogenous rhythms.^{54,55} The fact that the seasonal variation in susceptibility to the clinical signs of EAE was detected under controlled environmental conditions including a 12:12 hour light:dark cycle suggests that it may reflect an endogenous circannual rhythm rather than an exogenously controlled one. Using these criteria, the seasonal effect on clinical EAE can therefore be considered an intrinsic genetically controlled disease component. By inference, the seasonal effect in MS and monosymptomatic optic neuritis may also be because of an endogenous circannual rhythm rather than an exogenous rhythm dependent on environmental cues.^{54,55} Importantly, there is evidence indicating that endogenous circannual rhythms are genetically controlled.⁵⁵

The results of the linkage analysis to chromosome 17 marker loci indicate that the age and season at immunization significantly influence genetic linkage of clinical EAE to *eeae5*.^{20,21} Additionally, it suggests that the effect of these two factors may be because of two linked genes on chromosome 17 that independently modify clinical disease susceptibility. Importantly, these results argue for nonclassical major histocompatibility complex (MHC) loci because SJL/J and B10.S mice both possess the *H2^s*

haplotype²⁰ and linkage to chromosome 17 marker loci is distal of *H2-D*. In this regard, linkage to *HLA* is the single most consistent finding in all MS genetic studies.¹⁰ However, fine mapping studies have not definitively identified the *HLA*-linked MS susceptibility gene(s). Results suggesting that genes of interest in MS susceptibility reside within the class I, class II (particularly the *HLA-DR* locus⁵⁶), and class III regions^{57,58} of *HLA*, as well as telomeric of the class I region.^{59–62} Our results suggest that there may be multiple environmentally sensitive *HLA*-linked genes that contribute to MS. The existence of such genes may account for the varying linkage results observed across study populations and have the potential to represent examples of Simpson's paradox: a statistical phenomenon in which the effects at a locus can be masked, enhanced, or even reversed by gene-gene or gene-environment interactions that are not detected and accounted for.^{22–25} For complex traits such as autoimmune disease of the CNS, the implication is that it may not be possible to accurately predict and reproduce genotype-phenotype relationships if the most significant interactions underlying the system are incompletely characterized. For example, age- or season-associated changes in the expression of MHC molecules could influence susceptibility to EAE and MS as profoundly as MHC-restricted molecular mimicry.⁶³ Interestingly, the levels of both class I and class II molecules exhibit cell-specific changes in expression levels as a function of age.^{64–66}

Our data raise several considerations that should be taken into account with respect to forward genetic approaches aimed at identifying the gene or genes underlying the MHC-linked effect controlling CNS autoimmune disease. First, MS linkage studies using cohorts stratified by age and season at onset may significantly aid in delineating the *HLA*-linked MS gene or genes. Second, experimental approaches that rely on quantitative differences in gene expression to infer or establish causal relationships must account for age- and biological rhythm-based differences. Third, positional gene cloning approaches using congenic mapping as a strategy in EAE must take into account the effects of both age and season at immunization to accurately assess the degree of phenotypic variation ascribed to a given candidate interval or gene.

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