New Loci Regulating Rat Myelin Oligodendrocyte Glycoprotein-Induced Experimental Autoimmune Encephalomyelitis[1]

Kristina Becanovic,* Erik Wallstrom,* Barbara Kornek,‡ Anna Glaser,† Karl W. Broman,§ Ingrid Dahlman,* Peter Olofsson,¶ Rikard Holmdahl,¶ Holger Luthman,† Hans Lassmann,‡ and Tomas Olsson[2]*

Myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis (EAE) is an inflammatory disease in rats that closely mimics many clinical and histopathological aspects of multiple sclerosis. Non-MHC quantitative trait loci regulating myelin oligodendrocyte glycoprotein-induced EAE have previously been identified in the EAE-permissive strain, DA, on rat chromosomes 4, 10, 15, and 18. To find any additional gene loci in another well-known EAE-permissive strain and thereby to assess any genetic heterogeneity in the regulation of the disease, we have performed a genome-wide linkage analysis in a reciprocal (LEW.1AV1 × PVG.1AV1) male/female F_2 population ($n = 185$). We examined reciprocal crosses, but no parent-of-origin effect was detected. The parental rat strains share the $RT1^{av1}$ MHC haplotype; thus, non-MHC genes control differences in EAE susceptibility. We identified *Eae16* on chromosome 8 and *Eae17* on chromosome 13, significantly linked to EAE phenotypes. Two loci, on chromosomes 1 and 17, respectively showed suggestive linkage to clinical and histopathological EAE phenotypes. *Eae16* and *Eae17* differ from those found in previously studied strain combinations, thus demonstrating genetic heterogeneity of EAE. Furthermore, we detected a locus-specific parent-of-origin effect with suggestive linkage in *Eae17*. Further genetic and functional dissection of these loci may disclose critical disease-regulating molecular mechanisms. ***The Journal of Immunology,*** **2003, 170: 1062–1069.**

Multiple sclerosis (MS)[3] is a chronic inflammatory and demyelinating disease of the CNS causing neurological deficits. It is highly likely that autoimmune responses to CNS components are involved in the disease pathogenesis. There is a genetic influence in MS ($\lambda_{Sib} = 20$), perhaps most strikingly demonstrated in twin and adoption studies (1, 2). A prime motivation for finding genes regulating organ-specific inflammatory diseases such as MS is that these genes are likely to be crucial in the pathogenic pathway leading to disease, in turn with implications for new therapeutic strategies.

To date, whole genome scans of family materials and linkage analyses have failed to identify individual genes regulating MS, and, while certain genome regions overlap between the scans, many regions differ between the studies (3–6). Association studies in materials of sporadic cases and population-based controls have also largely failed to reveal consistent disease-regulatory genes, apart from the HLA complex and possibly CTLA-4 (7, 8). Many of the discrepancies between both the linkage studies and the association studies are likely to be explained by differing disease-associated genes between individuals and/or populations, i.e., genetic heterogeneity. Furthermore, the effect of any single gene on susceptibility may be modest, and individuals can be affected because of the cumulative effect of many genes, i.e., polygeneity. Very large materials of patients may therefore be needed to identify disease-predisposing genes. Some of these problems can be circumvented by the use of animal models of MS, such as experimental autoimmune encephalomyelitis (EAE) (9). First, genetic heterogeneity is minimized through the use of inbred susceptible and resistant strains. Second, there is no limitation of family sizes. Third, environmental triggers and conditions can be better controlled.

If polymorphic genes regulating disease are found in these models, they can be assessed for relevance in large materials of human MS cases compared with population-based controls. There is preliminary evidence that at least some genome regions identified experimentally may have impact on human MS (10–12). Even if the regulating genes are not the same in the different species, genetic dissection of animal models may reveal pathways of importance for human disease. To date, a limited number of rodent crosses have been analyzed to disclose disease-regulating non-MHC loci. The first linkage analysis in EAE was performed with an intercross between susceptible RIIIS/J mice and resistant B10.RIII mice (13). Since then, up to 20 mouse EAE quantitative trait loci (QTLs) have been identified (14, 15). In the rat, EAE QTLs have been demonstrated in DA × BN, LEW × BN, and

*Neuroimmunology Unit, Department of Medicine, and †Rolf Luft Center for Diabetes Research, Department of Molecular Medicine, Karolinska Institutet, Stockholm, Sweden; ‡Brain Research Institute, University of Vienna, Vienna, Austria; §Department of Biostatistics, Johns Hopkins University, Baltimore, MD 21205; and ¶Section for Medical Inflammation Research, Biomedical Center, Lund University, Lund, Sweden

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[2] Address correspondence and reprint requests to Dr. Tomas Olsson, Department of Medicine, Neuroimmunology Unit, Center for Molecular Medicine, Karolinska Hospital, 171 76 Stockholm, Sweden. E-mail address: Tomas.Olsson@cmm.ki.se

[3] Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; LOD, base 10 logarithm of the likelihood ratio; MOG, myelin oligodendrocyte glycoprotein; p.i., postimmunization; QTL, quantitative trait locus.

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E3 × DA intercrosses subjected to immunization with whole spinal cord homogenate (16–18) and with MOG as immunogen in DA × ACI and DA × PVG.1AV1 intercrosses (19, 20).

EAE can be induced in a variety of rodent species with a variety of myelin Ags. We believe that close mimicry of MS is important for the elucidation of mechanisms relevant for the human disease. Many EAE models are acute and monophasic, lacking important features of MS such as chronicity and demyelination. EAE induced with myelin oligodendrocyte glycoprotein (MOG) in certain strains of rats closely mimics MS histopathologically, with prominent demyelination and axonal damage, and has a chronic relapsing disease course (21–23). We have thus used MOG-EAE for the dissection of gene regulation of rat MS-like disease.

In previous studies of crosses between the MOG-EAE-permissive DA strain and the relatively MOG-EAE-resistant PVG.1AV1 or ACI strains, we have found loci which contribute to pathogenesis on rat chromosomes 4, 10, 15 and 18 (19, 20, 24). In view of the likely genetic heterogeneity observed in human MS, we next wanted to confirm the identified loci and/or find additional loci in a new cross, using the well-known EAE-permissive LEW strain crossed with the relatively resistant PVG strain. Because the MHC is already known to regulate MOG-EAE (21, 25), we selected the disease-susceptible LEW.1AV1 and the disease-resistant PVG.1AV1 strains, which share the rat MHC haplotype $RT1^{av1}$ (DA) for our cross. From the outset, we decided to establish a reciprocal cross with female/male founders of the respective strain, to be able to address parent-of-origin effects in the F_2 population. Such effects have been observed within whole F_1 and/or F_2 populations. In addition, further analysis may reveal locus-specific parent-of-origin effects (15, 26).

This study identifies loci regulating MOG-EAE (*Eae16–17*). No significant effects from previously identified loci on rat chromosomes 4, 10, 15, and 18 were detected. Thus, the LEW rat is MOG-EAE permissive for different genetic reasons than is the DA rat, demonstrating genetic heterogeneity concordant with what is speculated on in MS. Further fine mapping of these QTLs may help to characterize disease-relevant pathways.

Materials and Methods

Animal breeding

LEW.1AV1 and PVG.1AV1 were originally obtained from the Zentralinstitut fur Versuchstierzucht (Hannover, Germany) (27). All animals, both the parental and the F_2 intercross animals, were locally bred in light- and temperature-regulated rooms under specific pathogen-free conditions (with free access to water and food). The north Stockholm ethical committee approved the experiments. Parental LEW.1AV1 and PVG.1AV1 rats, F_1(LEW.1AV1 × PVG.1AV1), 96 F_2(LEW.1AV1 × PVG.1AV1) rats, and 89 F_2(PVG.1AV1 × LEW.1AV1) rats were used.

Induction and clinical evaluation of EAE

Rats between 8 and 11 wk of age were anesthetized with halothane and immunized intradermally in the tail base. Each rat received 200 μl inoculum containing 20 μg rMOG (aa 1–125) (28), mixed with 100 μl IFA (Sigma-Aldrich, St. Louis, MO) and 100 μl PBS (Life Technologies, Paisley, U.K.). Animals were weighed, and clinical signs of disease were evaluated from day 7 to day 40 postimmunization (p.i.). The signs were scored as follows: 1, tail weakness or tail paralysis; 2, hind leg paraparesis (gait disturbance) or hemiparesis; 3, hind leg para-paralysis or hemiparalysis; 4, tetraplegy, urinary and/or fecal incontinence. A relapsing/remitting disease was defined as a disease course in which the rats had remitted from maximum score 2–4 to 0 or 1, respectively (two scale units), for at least 2 consecutive days and then again relapsed for at least 2 days with maximum score 2–4. Monophasic disease course appeared as one bout of disease with recovery and no further signs of disease until day 40. Acute lethal disease was defined as disease with lethal outcome of the first bout. Primary progressive disease course was defined as constant and worsening nonremitting signs of disease. If severe balance disturbance and/or severe disease were observed for more than 1 day, the rat was sacrificed. Only rats with a body weight below 250 g on the day of immunization were included in the pheno- and genotypic analyses, to minimize threshold/immunization dose effects. Furthermore, our long-term experience with LEW rats of higher weight than 250 g is that disease incidence is more variable. EAE was defined as when the rat displayed clinical signs for more than 1 day, and onset was calculated as the first day the clinical signs were observed.

Histopathological evaluation

Rats were sacrificed and perfused via the left ventricle of the heart with 4% paraformaldehyde on day 40 p.i. Brains and spinal cords were dissected and embedded in paraffin wax. Sections 2–4 μm thick were cut on a microtome and stained with H&E, Luxol fast blue, and periodic acid-Schiff to assess inflammation and demyelination, respectively (22). Inflammation and demyelination were assessed on brain and spinal cord sections. To assess the extent of inflammation, the mean number of inflammatory infiltrates around vessels in the spinal cord was evaluated. To assess the degree of demyelination, a semiquantiative score slightly modified from that described by Storch et al. (22) was used. The scale ranged from 0 to 4 for brain and spinal cord, with a maximum score of 8 per animal. The scores were obtained as follows: 1 = perivascular/subpial demyelination; 2 = marked demyelination; 3 = extended demyelination, e.g., more than half of the spinal cord white matter or one optic nerve or more than half of the cerebellar white matter; 4 = full demyelination of the spinal cord white matter, or both optic nerves or the cerebellar white matter.

Anti-MOG IgG isotype determination

Serum was sampled from each rat on day 12 p.i. Anti-MOG IgG, IgG1, IgG2a, IgG2b, and IgG2c for each rat were determined by ELISA, as described (19). ELISA plates (Nunc, Roskilde, Denmark) were coated with 100 μl rat rMOG (aa 1–125) diluted in 0.1 M $NaHCO_3$, pH 8.2, to a concentration of 2.5 μg/ml. The coated plates were stored overnight at 4°C. The sera for measuring IgG, IgG2a, and IgG2b isotype levels were diluted 1/2000, and the sera for IgG1 and IgG2c were diluted 1/200. Antiserum was diluted as follows: IgG, IgG2a, and IgG2b, 1/2000; IgG1, 1/1000; IgG2c, 1/500 (Nordic, Tilburg, The Netherlands). Goat anti-rabbit conjugate was diluted 1/10,000 (Nordic). OD values were read at 450 nm. Each plate had DA serum (immunized with MOG) as a positive control and PVG.1AV1 serum as a negative control in duplicates. Arbitrary units were calculated for each rat and for each IgG isotype, by comparing the values with the standard curve of the positive control, the DA, for each ELISA plate.

Genotype analysis

Genomic DNA was prepared from tail tips according to a standard protocol (29). Microsatellite markers polymorphic for the LEW.1AV1 and PVG.1AV1 strains were used in a PCR-based amplification together with primers end labeled with [γ-^{33}P]ATP. Primers were obtained from GENSET (Paris, France). The PCR products were size fractionated on 6% polyacrylamide gels and visualized by autoradiography. Genotypes were determined manually and double checked. MAPMAKER/EXP, version 3.0, was used to create a genetic map. The coverage of the genome was 84% within 10 cM of a marker. The map was compared with an integrated map to calculate the chromosomal coverage (%) (kindly provided by H. Luthman, Department of Molecular Medicine, Karolinska Hospital; unpublished observations). RNO1, 89%; RNO_2, 96%; RNO3, 73%; RNO4, 78%; RNO5, 91%; RNO6, 89%; RNO7, 80%; RNO8, 91%; RNO9, 83%; RNO10, 88%; RNO11, 80%; RNO12, 88%; RNO13, 99%; RNO14, 73%; RNO15, 72%; RNO16, 95%; RNO17, 88%; RNO18, 97%; RNO19, 65%; RNO_20, 73%; RNOX, 78%

Statistical analysis

Linkage analysis and permutation tests were performed using the Map Manager QTXb15 software (http://mapmgr.roswellpark.org/mmQTX.html). Threshold values of the permutation test, which are labeled suggestive, significant, and highly significant, are derived from the guidelines of Lander and Kruglyak (30–32) and correspond to the thresholds representing 0.63, 0.05, and 0.001 for a complete genome scan. Permutation tests are used to determine significance levels based on the analyzed sample material (33, 34). Permutation analysis involves repeated shuffling of the trait values 1000 times among the genotypes to calculate relevant significance levels. The same method was used by Lander and Kruglyak, but on a totally computerized and randomized material. The permutation procedure based on the investigated material is empirical and reflects the characteristics of the particular experiment to which it is applied. This method does not rely on distributional assumptions regarding the quantitative trait, and is valid in small sample situations (34). Threshold levels for significance

Table I. *Clinical EAE parameters measured in LEW.1AV1, PVG.1AV1, F_1, and F_2 rats*

Strain/Cross	Incidence (%)[a]	Gender and Incidence[a]	Maximum Clinical Score 0	1	2	3	4	Mean Maximum EAE Score ± SD[b]	Mean Onset Day of Disease ± SD[c]	Mean Maximum Weight Loss ± SD[c,d] (%)
LEW.1AV1	40/48 (83 %)	♀ 25/26 ♂ 15/22	8	1	13	12	14	2.5 ± 1.4	15 ± 6.0	17% ± 7.1
PVG.1AV1	4/61 (6.6 %)	♀ 1/29 ♂ 3/32	57		2	2		0.16 ± 0.64	23 ± 6.4	−4.9% ± 7.7
(LEW.1AV1 × PVG.1AV1)F_1	32/52 (62 %)	♀ 23/27 ♂ 9/25	20	2	2	14	14	2.0 ± 1.7	15 ± 4.4	19% ± 8.2
F_2 intercross	132/185 (71%)	♀ 107/142 ♂ 25/43	53	1	15	73	43	2.3 ± 1.6	16 ± 4.5	8.4% ± 9.5

[a] Number of affected rats/total number of rats.
[b] Mean values are calculated from affected and nonaffected rats.
[c] Mean values are calculated from affected rats only.
[d] Mean change in body weight days 7–40.

were assessed by permutation analysis for the following phenotypes on our material. Threshold level for suggestive linkage = base 10 logarithm of the likelihood ratio (LOD) 2.3 and significant linkage = LOD 3.7 determined for maximum EAE score (M), incidence (I), demyelination score (DM), demyelination score standardized, weight loss (W) standardized, and anti-MOG Ab (A) IgG, IgG1, and IgG2c. Threshold level for suggestive linkage = LOD 2.3 and significance linkage = LOD 3.8 for cumulative EAE score (C) and weight loss (W). These phenotypes are likely to be coupled, which has to be considered with regard to the multiple comparisons performed. Corrections for multiple comparisons (gender and reciprocal crosses) were made using the Bonferroni method. In addition, Fisher's exact test was used to analyze whether there was a difference in observed genotype distribution between affected and nonaffected rats, i.e., rats with positive clinical score. Kruskal-Wallis ranking test was used to determine whether different genotypes were associated with differences in maximum EAE score, cumulative EAE score, weight loss, demyelination, inflammation, and anti-MOG Ab levels. The Mann-Whitney test was used to compare the anti-MOG IgG levels between affected and nonaffected rats. We have tested for influence of gender on the EAE phenotypes, on which we performed linkage analysis. The gender effect was considered significant for weight loss and demyelination ($p \leq 0.01$). The phenotypic values were therefore standardized using the z-transformation, with the formula: $z_x = (x-\mu_x)/\sigma_x$. Both standardized and nonstandardized values were analyzed, and results are shown in Table II.

Results

Clinical disease

Table I displays the incidence of clinical signs of EAE in LEW.1AV1, PVG.1AV1, F_1, and F_2 progeny immunized with rMOG (aa 1–125) in IFA. As expected, LEW.1AV1 displayed a high incidence of EAE with 40 of 48 (83%), while only 4 of 61 PVG.1AV1 rats (6.6%) developed clinical signs of disease and with delayed onset. F_1 rats displayed an intermediate EAE phenotype (62% incidence), excluding any highly dominant or recessive mode of inheritance. A total of 132 (71%) F_2 rats displayed a wide spectrum of clinical signs of EAE. The disease course mimicked the clinical spectrum of MS, with acute lethal (LEW.1AV1, 25%; PVG.1AV1, 0%; F_1, 0%; F_2, 2.2%), monophasic (LEW.1AV1, 19%; PVG.1AV1, 6.3%; F_1, 7.7%; F_2, 9.7%), relapsing/remitting (LEW.1AV1, 33%; PVG.1AV1, 0%; F_1, 29%; F_2, 24%), and primary progressive (LEW.1AV1, 6.3%; PVG.1AV1, 3.2%; F_1, 25%; F_2, 34%) disease courses. Some rats developed balance disturbance (LEW.1AV1, 8.3%; PVG.1AV1, 1.6%; F_1, 23%; F_2, 3.4%). We observed a higher incidence of EAE and means of maximum score, respectively, in females compared with males in LEW.1AV1 rats ($p = 0.013$; $p = 0.00021$, Fisher's exact test and Mann-Whitney U test rank sums test, respectively), F_1 ($p = 0.0003$; $p = 0.0002$), and F_2 ($p = 0.025$; $p = 0.039$).

Histopathological evaluation

Inflammation and demyelination were semiquantitatively assessed in brain and spinal cord sections day 40 p.i. These phenotypes were largely distributed in a similar way as clinical signs of disease, with high demyelination and inflammation indices among parental LEW.1AV1 rats, little among PVG.1AV1 rats, and intermediate patterns in F_1 and F_2 rats (Fig. 1). The inflammation indices were relatively low compared with the demyelination scores, most apparent among the F_1 and PVG.1AV1 parental rats. This is

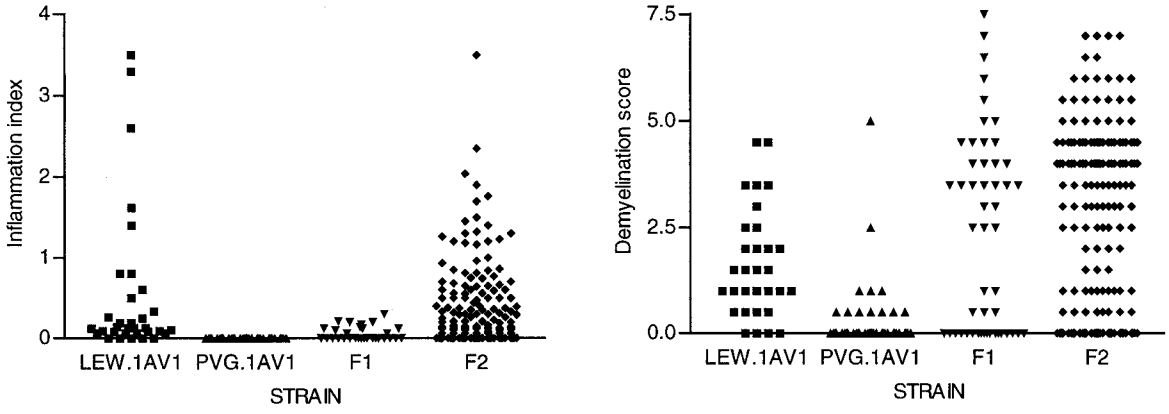

FIGURE 1. Semiquantitative histopathological assessments of MOG-EAE day 40 p.i. Degree of inflammation and demyelination in parental strains and the F_1 and F_2 progeny. To assess the extent of inflammation, the mean number of inflammatory infiltrates around vessels in the spinal cord was evaluated. To assess demyelination, a semiquantitative score ranging from 0 to 4 for brain and spinal cord, with a maximum demyelination score of 8 per animal (for details, see *Materials and Methods*).

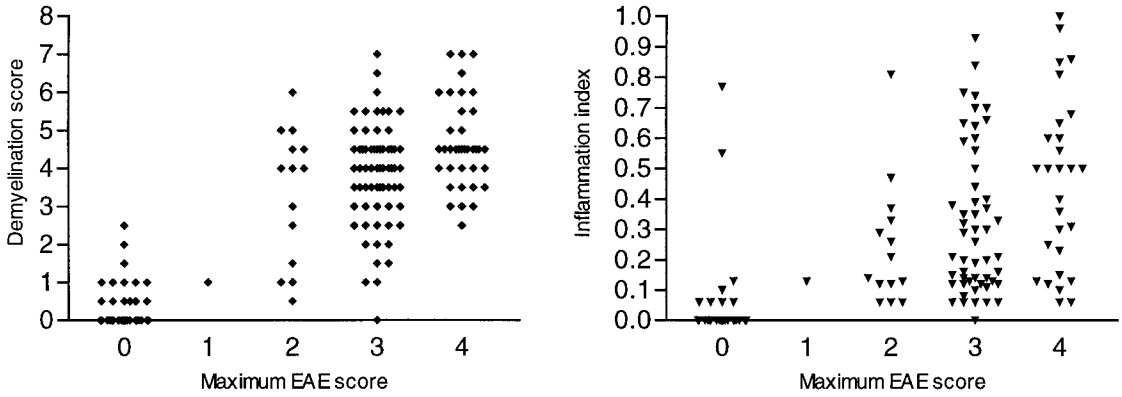

FIGURE 2. Maximum EAE score plotted against histopathological phenotypes assessed day 40 p.i. reveals correlation with demyelination score (Spearman $r = 0.75$, $p < 0001$) and inflammation index (Spearman $r = 0.72$, $p < 0.0001$).

expected in view of the late sampling time day 40 p.i. in this study, a time point when T cell and macrophage infiltration has decreased compared with early in disease course (21, 22). Still, maximum EAE score correlated with both degree of inflammation and demyelination (Fig. 2). There were 17 rats among the 185 F_2 rats selected for genotyping that displayed histological lesions, but no overt clinical signs. This suggests a subclinical healed disease with histological sequelae, a subclinical active disease process, and/or signs not assessed in our scoring procedure, such as sensory deficits.

Humoral immune response

We measured anti-MOG Ab serum levels day 12 p.i. This phenotype may reflect both the T and B cell arms of MOG autoimmunity, because T cell help is needed for B cell production of anti-MOG Ab (35, 36). In addition, we measured anti-MOG IgG isotypes, which could potentially discriminate between a T1/T2 bias in the immune response. IgG2b and IgG2c are associated with T1, and IgG1 is associated with T2 responses in the rat (37). Because anti-MOG Abs may be important effectors in demyelination, we studied whether there were any correlations between the different IgG isotypes and this histopathological phenotype. Weak correlations in the F_2 population were found for anti-MOG IgG2c and IgG1 (Fig. 3). Affected compared with nonaffected F_2 rats displayed higher total IgG ($p < 0.0001$, Mann-Whitney test), IgG1 ($p = 0.0058$), IgG2a ($p = 0.0003$), IgG2b ($p = 0.0050$), and IgG2c levels ($p = 0.0010$) (Fig. 4).

Reciprocal crosses

To study whether there were any parent-of-origin effects, we bred reciprocal crosses between susceptible LEW.1AV1 and the relatively resistant PVG.1AV1 rats to produce two types of F_1 progeny: 15 (LEW.1AV1 female $\times$ PVG.1AV1 male)F_1 rats and 37 (PVG.1AV1 female $\times$ LEW.1AV1 male)F_1 rats, of which 11 of 15 (73%) and 21 of 37 (57%) rats, respectively, developed EAE after immunization. The two types of progeny were then separately intercrossed to produce F_2 progeny for the whole genome scan described below. A total of 69 of 96 (72%) (LEW.1AV1 female $\times$ PVG.1AV1 male)F_2 and 63 of 89 (71%) (PVG.1AV1 female $\times$ LEW.1AV1 male)F_2 rats developed clinical signs of EAE. Subgroup analysis did not display any differences in maximum EAE score or EAE incidence when comparing the females and males, respectively, in the F_1 and F_2 progenies having a LEW.1AV1 or PVG.1AV1 female founder. Combined data are shown in Table I.

Genetic mapping and linkage analysis

A total of 185 F_2 rats were genotyped with a total of 235 microsatellite markers distributed throughout the rat genome (with an approximate distance between markers of 10–20 cM). Linkage analysis and permutation analysis were performed using the software Map Manager QTXb15 (http://mapmgr.roswellpark.org/mmQTX.html) (31, 32). Loci with evidence of linkage were mapped with additional adjacent markers. We further analyzed the

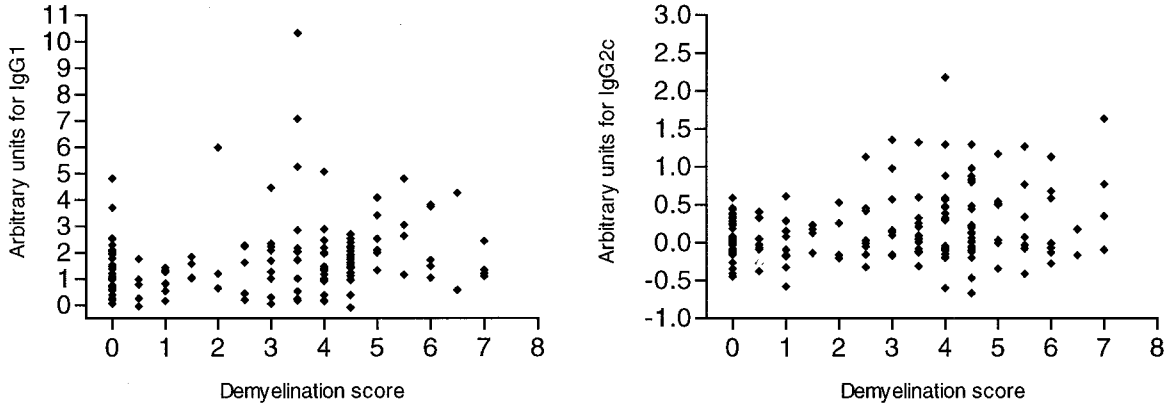

FIGURE 3. Demyelination score assessed histopathologically day 40 p.i. plotted against serum anti-MOG Ab levels day 12 p.i. reveals a weak correlation with IgG1 (Spearman $r = 0.27$, $p = 0.0014$) and IgG2c Ab levels (Spearman $r = 0.22$, $p = 0.0033$).

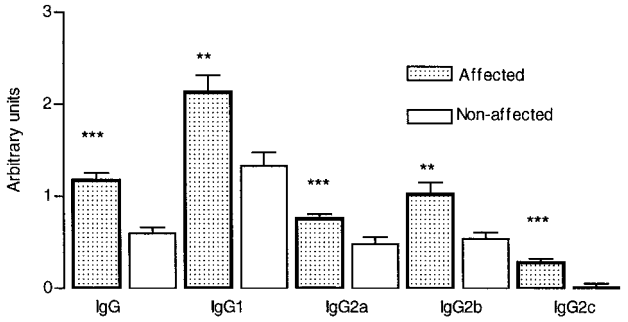

FIGURE 4. Serum anti-MOG Ab levels measured by ELISA day 12 p.i. in (LEW.1AV1 × PVG.1AV1)F_2 progeny. Arbitrary units obtained from a standard curve of a positive sample run in parallel are given. Affected compared with nonaffected F_2 rats displayed significantly higher total anti-MOG IgG, IgG1, IgG2a, IgG2b, and IgG2c levels ± SEM. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

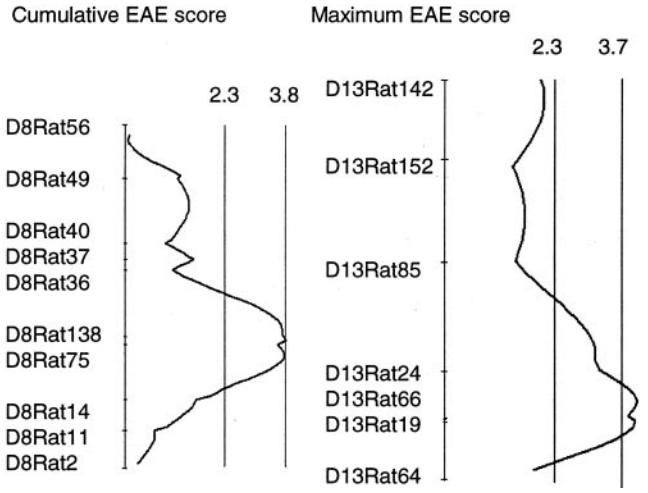

FIGURE 5. Log-likelihood plots of QTLs identified in a (LEW.1AV1 × PVG.1AV1)F_2 intercross. There is significant linkage for cumulative EAE score on chromosome 8, and maximum EAE score on chromosome 13. The vertical lines indicate suggestive and significant threshold levels determined by permutation analysis performed on the F_2 material. Marker scale ~24 cM/cm and ~14 cM/cm for chromosomes 8 and 13, respectively.

loci identified in the linkage analysis in subgroups to assess gender differences and parent-of-origin effects.

We identified one locus on chromosome 8, named *Eae16*, significantly linked to regulation of cumulative EAE score (Table II and Fig. 5). This phenotype is complex and includes EAE incidence, maximum EAE score, and duration. Assessed individually, these phenotypes displayed *p* values of $p = 0.051$, $p = 0.024$, and $p = 0.005$, respectively (Kruskal-Wallis test). The anti-MOG response was not affected. The LEW alleles are disease enhancing in a recessive manner.

Eae17 on chromosome 13 displayed significant linkage to maximum EAE score and incidence (Table II and Fig. 5). Further analysis demonstrated suggestive linkage for this effect within the group of animals with PVG.1AV1 as female and LEW.1AV1 as male founders (Fig. 6). Rats homozygous for PVG alleles in this group had significantly lower mean maximum EAE scores compared with the other two genotype groups. Sex-segregated QTL analysis of this locus indicated that females alone displayed significant linkage to maximum EAE score at marker D13Rat19, ~12 cM on chromosome 13 (LOD 4.0). This peak marker was the same as when we performed the linkage analysis with all F_2 animals. However, male rats displayed a peak with suggestive linkage to maximum EAE score at ~73 cM (LOD 3.5) (Fig. 7). We also detected effects of this QTL on EAE incidence (Table II).

We identified one locus on chromosome 1 with suggestive association to maximum weight loss and clinical and histological phenotypes (Table II). Weight loss is considered as an EAE-related quantitative trait, because it precedes and then parallels clinical EAE signs. LEW alleles in this locus are disease predisposing in a dominant fashion.

Table II. *Summary of major QTLs detected in a (LEW.1AV1 × PVG.1AV1)F_2 intercross*[a]

QTL[b]	Chromosome	Peak Marker	Phenotype[c]						Inheritance Pattern[d]
			M	I	C	DM	W	A	
	1	D1Rat4		3.0	2.7	2.8[f]	2.7[f]		LEW dominant
	1	D1Rat250[e]	2.7				3.6[f]		LEW dominant
	1	D1Rat126				3.1			
Eae16	8	D8Rat75			3.8*				LEW recessive
	8	D8Rat36						3.5[g,h]	PVG additive
Eae17	13	D13Rat19	4.0*	4.1*					LEW dominant
	13	D13Rat24[e]						3.4[g,i]	Heterozygous> LEW, PVG
	17	D17Rat67	3.2	2.8		3.1[f]			LEW additive
	19	D19Rat2						2.8[g,j]	Heterozygous> LEW, PVG

[a] Linkage analysis and permutation analysis were performed with Map Manager QTXb15 (http://mapmgr. roswellpark.org/mmQTX.html). *, LOD value with genome-wide significance. No asterisk denotes suggestive linkage.

[b] EAE modifying QTLs are numbered in order of discovery, *Eae-n.*

[c] LOD values for different EAE phenotypes; threshold level for suggestive linkage = LOD 2.3 and significant linkage = LOD 3.7 for maximum EAE score (M), incidence (I), demyelination score (DM), demyelination score standardized, weight loss (W) standardized, and anti-MOG Ab IgG, IgG1, and IgG2c (A). Suggestive linkage = LOD 2.3 and significant linkage = LOD 3.8 for cumulative EAE score (C) and weight loss (W).

[d] Inheritance pattern with regard to the strain displaying the highest mean of the trait.

[e] Distance between D1Rat4 and D1Rat250 is 4.5 cM. Distance between D13Rat19 and D13Rat24 is 9 cM.

[f] When the trait is standardized for gender, LOD score for weight loss on D1Rat250 and D1Rat4 is 3.2 and 2.7, respectively. LOD score for demyelination on chr.1 and chr.17 is 2.6 for D1Rat4 and 3.6 for D17Rat67, respectively (z-transformation of traits is performed, as described at http://www.animatedsoftware.com).

[g] Logarithmic values were used for the analysis of serum levels of anti-MOG Ab isotypes.

[h] The locus is linked to anti-MOG IgG1 isotype Abs.

[i] The locus is linked to anti-MOG IgG2c isotype Abs.

[j] The locus is linked to anti-MOG IgG isotype Abs.

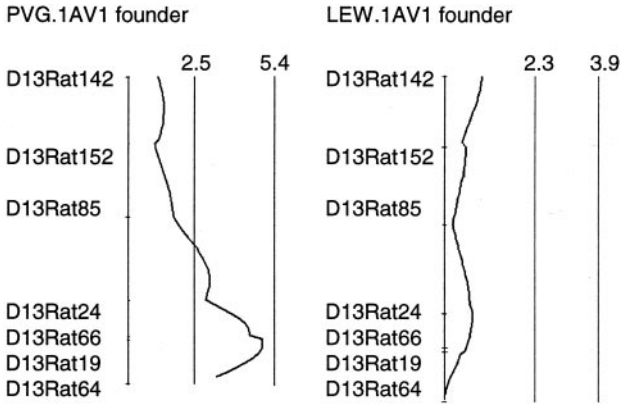

FIGURE 6. Parent-of-origin-segregated log-likelihood plots of *Eae17* on rat chromosome 13. Suggestive linkage to maximum EAE score was restricted to (PVG.1AV1 female × LEW.1AV1 male)F_2 rats (LOD 5.0). Vertical lines within a graph represent suggestive and significant linkage cutoff levels for maximum EAE score determined by permutation tests performed on the segregated F_2 material (PVG.1AV1 female founder, n = 89; LEW.1AV1 female founder, n = 96). Marker scale ~17 cM/cm.

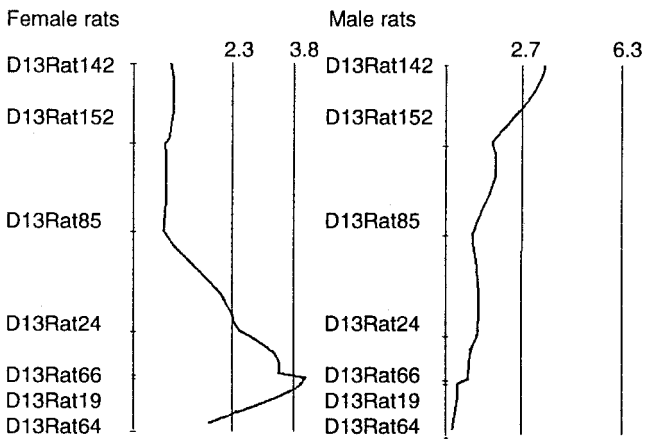

FIGURE 7. Sex-segregated log-likelihood plots of *Eae17* on rat chromosome 13. Vertical lines within a graph represent suggestive and significant linkage cutoff levels for maximum EAE score determined by permutation tests performed on the segregated F_2 material (males, n = 43; females, n = 142). Marker scale ~15 cM/cm. Females alone displayed significant linkage (LOD 4.0) at marker D13Rat19, ~12 cM. Male rats displayed suggestive linkage (LOD 3.5) to maximum EAE score at marker D13Rat142, ~73 cM.

One locus on chromosome 17 also displayed suggestive linkage to both clinical and histological phenotypes (Table II). This locus was evident as a quite broad peak, with several markers ~5 cM apart displaying significant linkage. LEW alleles are disease predisposing, and the inheritance pattern is additive.

Determination of serum anti-MOG Ab IgG1, IgG, and IgG2c isotype levels day 12 p.i. revealed signs of regulation from regions on chromosomes 8, 19, and 13, respectively (Table II). Although the chromosome 13 locus also affected clinical disease phenotypes, as described above, the anti-MOG Ab-regulating loci on chromosomes 8 and 19 did not.

Discussion

In this study, we present data from a genome-wide linkage analysis of a reciprocal F_2 intercross between the EAE-permissive LEW.1AV1 and the relatively resistant PVG.1AV1 rat, which provides new information about strain-dependent heterogeneity and polygeneity in the non-MHC gene regulation of MOG-EAE. Two EAE-associated QTLs were identified (Fig. 5). *Eae16* on chromosome 8 was linked to cumulative EAE score, and *Eae17* on chromosome 13 was linked to maximum EAE score, incidence, and anti-MOG IgG2c Ab levels. In addition, two loci on chromosomes 1 and 17 displayed suggestive linkage to weight loss and demyelination, respectively. *Eae16* and *Eae17* differ from previously identified MOG-EAE-regulating loci using other rat strain combinations (Fig. 8) or mouse EAE models. This demonstrates that the genetic regulation of MOG-EAE, and probably EAE in general, is

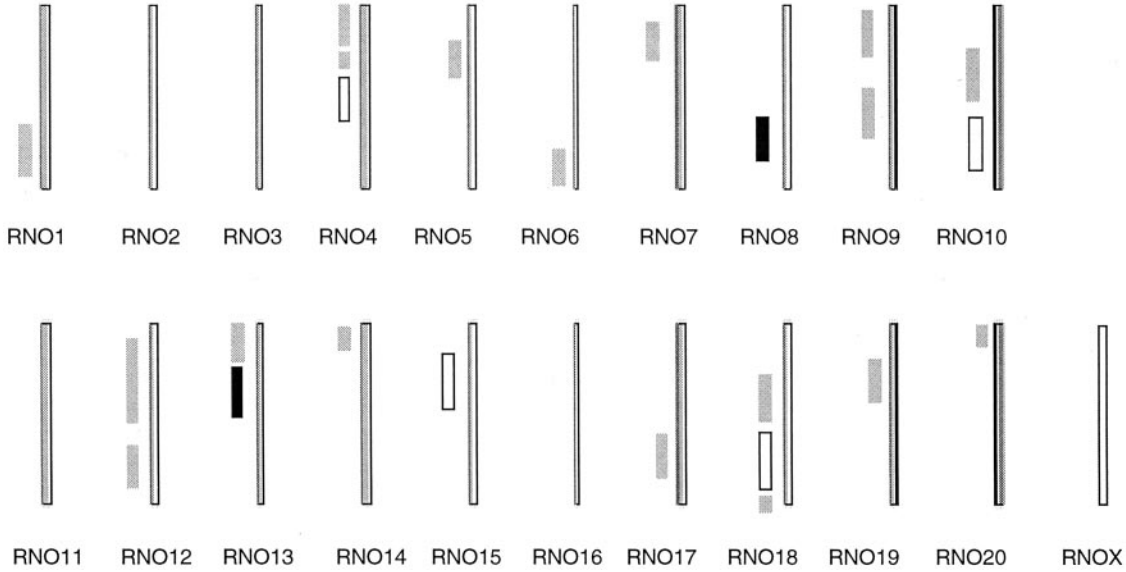

FIGURE 8. Summary of EAE loci identified in rat. RNO (*Rattus norvegicus)* and the chromosome numbers are displayed. *Eae16* and *Eae17* identified on chromosomes 8 and 13, respectively, were identified in the herein described (LEW.1AV1 × PVG.1AV1)F_2 intercross and are represented by black boxes. Previous MOG-EAE loci identified in DA × ACI and DA × PVG.1AV1 intercrosses (16, 20) are represented by white boxes. Other identified loci in other cross combinations and with different induction protocols are indicated with gray boxes (17, 18). Because proper confidence intervals are lacking for many of the previously reported QTLs, they are depicted in their approximate positions as based on linkage maps available from http://ratmap.ims.u-tokyo.ac.jp/cgi-bin/comparative_home.pl, http://www.informatics.jax.org/.

heterogeneous in a similar manner to what is strongly suspected in human MS (38–40). The observation of several new disease loci not previously found in other rat or mouse EAE models (14, 41) is most likely caused by variations in pathogenesis between different models and by species/strain differences. In addition to these new loci, we demonstrate a possible locus-specific parent-of-origin interaction in rat EAE.

Apart from the specific loci discussed below, other noteworthy observations also emerged from correlation studies of different subphenotypes in the F_2 cross. Thus, the overall systemic humoral immune response against MOG in the form of anti-MOG Ab serum levels correlated with both clinical and histopathological measures such as inflammation index and demyelination. We found no overt bias in the genetic regulation of the T1/T2 differentiation as measured by Ab isotypes, consistent with the hypothesis that MOG-EAE in this setting may depend on both lymphocyte differentiation patterns (42). This is in contrast to our findings in a recently published linkage analysis of experimental autoimmune neuritis in (DA $\times$ ACI)F_2 rats, in which a genetically regulated systemic Ab response reflecting a T1 bias correlated with more pronounced disease (43).

Disease-regulatory genes may influence disease incidence and/or severity/chronicity. There is experimental evidence supporting the notion that certain QTLs specifically enhance disease severity and/or chronicity, while not affecting the disease incidence (18). *Eae16* belongs to this category, as it displayed association with disease severity and duration, but not incidence. Theoretically, cumulative EAE score may be a less accurate phenotype because the scale intervals in the clinical EAE grading are not proportional to each other. In practice, rats with severe disease also have disease of longer duration. Severity is also one of the most important parameters in patients with MS, with 5–20% having benign disease and never developing disabling neurological deficits (44), suggesting the presence of gene alleles specifically predisposing for severity of disease. In clinical medicine, most resources have been focused on finding genes associated with development of MS rather than disease severity. If genetically regulating mechanisms for severe disease courses could be identified, they would potentially be prime therapeutic targets.

The chromosome 13 locus *Eae17* displayed linkage to maximum EAE score and EAE incidence. In a previous DA $\times$ ACI intercross, we identified a QTL in this region with suggestive linkage to EAE incidence and the histopathologically determined inflammation index (19). The identification of *Eae17* suggests disease-regulatory effects depending on parent-of-origin influence. Thus, this locus could interact and/or be activated as a disease-promoting locus, dependent on the origin of the animal. Similar effects were recently described in mouse EAE (15). This reciprocal cross effect can result from genetic factors, which could include mitochondrial genes, imprinted genes, or other factors. We have no possibility at this moment to discriminate between these possibilities. The finding has also to be regarded with some caution in view of multiple comparisons made. We also detected that the females displayed the peak at ~12 cM in contrast to the males, which displayed the peak at ~73 cM on chromosome 13. Similar gender effects have been reported before in rat experimental arthritis (45, 46). Reproduction and experiments are underway in our laboratory to test the influence of this genome region in reciprocal congenic strains.

The chromosome 1 locus displayed suggestive association to maximum weight loss, clinical EAE phenotypes, and demyelination. The histopathological features of the disease are likely to depend on the intensity of the inflammatory attack on the CNS, which in turn is correlated to the degree of clinical disease. However, the anti-MOG Ab serum levels were not significantly associated with this QTL, suggesting that the overall immune response to MOG was not an explanation for the genetic impact of this QTL. Instead, qualitative differences in the immune response or CNS target factors could be considered. The location of the chromosome 1 locus differed from previously defined loci in rat inflammatory diseases, such as a locus regulating whole spinal cord-induced EAE in a E3 $\times$ DA cross (18), although it colocalizes with a locus-regulating severity in pristane-induced arthritis (47).

The locus on chromosome 17 displayed suggestive linkage to demyelination, maximum EAE score, and EAE incidence. This locus colocalized with a locus described in a (LEW $\times$ BN)F_2 intercross with suggestive linkage to inflammation, and in which EAE was induced with whole spinal cord homogenate (17).

It is to some extent paradoxical that we found an overall correlation between systemic anti-MOG Ab levels and disease in the F_2 progeny, while Ab levels dissociated from regulation of clinical disease, at discrete QTLs. However, the QTL on chromosome 13 showed linkage to both clinical disease and anti-MOG IgG2c levels. These findings are consistent with those in whole spinal cord-induced disease in a DA $\times$ E3 F_2 intercross, in which loci-regulating disease and anti-MOG Abs also dissociated (48). Our interpretation is that the most conspicuous disease-regulating locus on chromosome 8 regulates features in the autoaggressive response not linked to quantitative aspects of the anti-MOG response. The overall correlation between the clinical outcome and the systemic anti-MOG immune response may, in addition to the chromosome 13 locus, be explained by a number of other loci, below the threshold of detection in this linkage analysis, which regulate both disease and the anti-MOG response.

In this study, we demonstrate experimental evidence for several factors either known to affect, or discussed in the context of, complex disorders in humans. These are polygeneity, genetic heterogeneity, and gene origin effects.

The identified loci can be explored using a variety of methods, including breeding of reciprocal congenic rat strains (49), the use of advanced intercross lines allowing a higher resolution mapping of the influences (50), as well as unbiased gene expression methodologies in congenic strains (51). All these approaches are currently underway in our lab.

In conclusion, we report a linkage study of a MS-mimicking rat model. The data may pave the way for definition of genetically regulated pathways in neuroinflammatory diseases. This in turn may lead to new therapeutic avenues with potential application for human disease.

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