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Genetics of experimental autoimmune encephalomyelitis in the mouse

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Summary

Multiple sclerosis (MS) is an inflammatory, demyelinating disease in the central nervous system (CNS) affecting approximately 0.1% of the population in the northern part of the world. The factors behind the initiation of the inflammatory response are not known at present, but MS is considered as a complex disease depending on genetic as well as environmental factors. Experimental autoimmune encephalomyelitis (EAE) is the prevailing experimental rodent model for multiple sclerosis (MS). Disease is induced in genetically susceptible mice or rats by immunization with myelin proteins or peptides, which leads to an infiltration of leukocytes into the CNS. EAE has been subjected to investigations of genetic susceptibility to disease development. By the identification of genes predisposing to EAE, the hope is to get clues as to what genetic elements are also important in MS. To date, more than 25 *Eae* loci have been described in the mouse. The quantitative trait loci are linked to different disease traits and several show sex specificity. Here we discuss the current state of the genetics controlling susceptibility to EAE.

Key words:

autoimmunity • experimental models • genetics • EAE • multiple sclerosis

Abbreviations:

EAE – experimental autoimmune encephalomyelitis, **MS** – multiple sclerosis, **QTL** – quantitative trait locus, **APC** – antigen-presenting cell, **MBP** – myelin basic protein, **PLP** – proteolipid protein, **MOG** – myelin oligodendrocyte glycoprotein.

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Autoimmune diseases comprise a number of disorders that affect approximately 5% of the population²⁷. Examples of autoimmune diseases are type I diabetes, rheumatoid arthritis (RA), Graves's disease, multiple sclerosis (MS), systemic lupus erythematosus (SLE), and a number of other organ-specific or systemic inflammatory diseases. Although autoimmune disorders affect different organs or are considered to be systemic, a common theme is an uncontrolled immune response towards self antigens that subsequently develops into chronic disease. Development of an autoimmune disease is controlled by both environmental and genetic factors⁴⁵. From genetic studies of autoimmune diseases in human cohorts and in experimental animal models it is known that these diseases are polygenic and that they are promoted by a number of genes that make small contributions to the overall disease outcome. To date it has been difficult to find genes that are significantly linked to susceptibility to autoimmune disease. This is due to several factors, such as the genetic diversity of the human population, influence of the environment, possible variations in the diagnosis of disease, and the relatively small sample sizes. The major histocompatibility complex (MHC) is strongly associated to the development of almost all autoimmune diseases (reviewed in¹⁸). It is, however, not completely elucidated how genes within this complex contribute to susceptibility or resistance to disease. There is strong data showing the importance for antigen presentation and the interaction between the antigen-presenting cell (APC) and the T cell receptor^{2, 40}. However, the role for other genes must be considered due to the large number of closely located genes within this complex.

To investigate the mechanisms behind the development of autoimmune diseases and how the disease course could be manipulated, animal models have been extensively studied. The advantage of studies in animal models, compared with humans, is that the genetic background and the environment are better under control. One disadvantage of the animal models is that they do not completely display all the disease parameters found in the human disease. However, it can be argued that specific traits of animal models reflect a particular pathway and give a better understanding of a particular stage of the human disease.

Investigations of the genetic contribution to the development of disease can be accomplished in two directions. One direction is to perform the studies from gene to disease. The other strategy is based on studies of disease development followed by genetic linkage analyses. The "gene-to-disease" strategy is

represented by the use of transgenic models, where one particular gene has been knocked out by homologous recombination. Subsequently, the investigation aims at understanding the role of the particular gene in the development of the disease in an autoimmune model. With this approach, which normally leads to a complete absence of the particular protein, one does not take into account the potential interactions between the absent protein and other proteins that make up the complexity of factors contributing to disease. In some experiments this has led to different results when the effect of a particular gene was investigated in different mouse strains and, thus, different genetic contexts^{26, 49, 52}.

The second strategy is to relate disease development to a genetic variant at a defined genetic location with the help of statistical linkage analysis. One strain of mice or rats that is susceptible to disease is crossed with a second, resistant strain, making F1 hybrids of the two strains. Subsequently, the F1 animals are crossed with each other or back-crossed to one of the parental strains, and a large number of offspring with segregating genes from both parental strains, are generated. Disease development is investigated and followed by a genome screen of all individuals in the cross with a large number of genetic markers. In this way different traits of the disease can be statistically linked to particular genetic regions. From an initial genetic scan, followed by linkage analysis, one normally ends up with a region comprising 10–20 centimorgan (cM) significantly linked to the disease trait. This corresponds to approximately 10–30% of the chromosome and needs to be considerably narrower in order to find candidate genes. By fine mapping the linked genetic regions with a large number of markers and by breeding congenic mice, these quantitative trait loci (QTL) can be further limited. When the QTL is 1–2 cM, approximately $1-2 \times 10^6$ base pairs, and a few candidate genes persist, those can be investigated for genetic polymorphisms between the parental strains. In addition, they could be investigated for differences on the transcriptional or protein levels. The "disease-to-gene" strategy is depicted in Fig. 1.

MS AND EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

The disease process in MS is characterized by autoimmune activity leading to the formation of sclerotic plaques in the central nervous system (CNS), focal inflammation followed by demyelination, and loss of axons. The MS pathogenesis eventually gives rise to a panel of different CNS-related signs and symptoms in the patient, a majority of the cases starting with a relapsing/remitting disease course followed

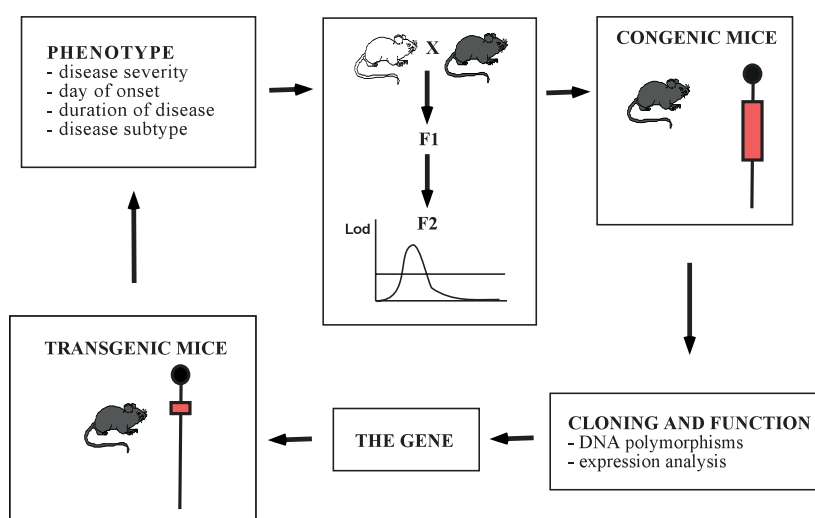


Figure 1. Gene-finding strategy. The disease phenotype is identified and sub-divided into disease traits that can be studied in animal crosses. Breeding of susceptible and resistant mouse strains will result in offspring with segregating genes. The disease phenotype is observed in the offspring and subsequent genotyping of all chromosomes in the animals is followed by statistical linkage analysis. Genetic regions that are significantly linked to any disease trait are bred onto the genetic background of one of the parental strains to establish congenic mice. By creating mice with small congenic fragments that still control the phenotype, it will be possible to find a small number of candidate genes that can be investigated for polymorphisms and for differential expression by comparing the congenic and the wild-type mice. When the important gene is cloned, it will be necessary to finally confirm the disease phenotype in transgenic animals.

by a progressive phase^{13, 23, 35}. The prevalence of MS varies across the globe, however: in the northern parts of the world the incidence is around 0.1%⁵⁷.

MS is a complex disease, depending on both genes and environment. In addition, susceptibility to MS is higher in women than in men. The concordance rate between monozygotic twins in MS is approximately 25–30%. As in other autoimmune diseases, there is a genetic association to the MHC^{20, 21, 46, 60}. Several studies in human MS families have been performed in search of genetic determinants, but linkage analyses have yielded no significant results so far. A number of candidate genes have been suggested from different studies and will be discussed in the context of what has been found in the experimental models^{8, 14, 24, 39, 67}.

The prevailing animal model, experimental autoimmune encephalomyelitis (EAE), has a number of features in common with MS^{61, 63} and has been extensively studied for many years in the search for mechanisms behind the development of human inflammatory disease. EAE is induced in susceptible mouse or rat strains by immunization with spinal cord homogenate (SCH), whole protein or peptides from myelin basic protein (MBP), myelin proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG). The immunization protocol normally includes adjuvant in the form of mycobacteria-supplemented oil, and in many models the induction of EAE is accomplished only by additional injections containing pertussis toxin. The disease course

involves paralysis of the tail and hind limbs and varies between mouse strains and immunization protocol. The different traits that can be recorded during the course of disease include day of onset, severity, and clinical subtype of EAE.

The process in EAE pathogenesis starts with the activation of CNS antigen-specific T cells in the peripheral immune system. In several studies, the presence of myelin-reactive T cells in healthy animals has been demonstrated³⁸. The central role of T cells is shown through experiments where disease can be transferred to naïve animals by myelin antigen-specific T lymphocytes⁷³. Once activated, the T cells gain access to the CNS via adhesion mechanisms on endothelial cells in the brain and migrate across the blood brain barrier. Within the CNS, after reactivation by resident APCs, the T cells produce cytokines that influence other immune cells, such as macrophages/microglia, B cells, and mast cells, to exert their effector functions⁶². The inflammatory response and the release of inflammatory mediators mediate destruction of the myelin sheath^{36, 55}. With this brief description of a complex disease process we want to point out that the pathogenesis of EAE and MS most likely work on many different levels and that many different genes are presumably involved.

IDENTIFICATION OF QTL IN DIFFERENT MOUSE EAE MODELS

Before discussing different *Eae* loci, it should be noted that there is at present incomplete consensus

between how the *Eae* loci are denoted in the literature and in the mouse genome database (<http://www.informatics.jax.org>). In this review we have chosen to use the *Eae* loci numbers reported in the literature.

Most of the QTL for the development of EAE have been identified in crosses between the SJL/J and B10.S mouse strains (*Eae4–Eae25*). In this model, chronic relapsing EAE is induced by immunization with SCH³⁷ or with the PLP peptide 139-151¹⁶. Some of the reported *Eae* loci (*Eae2*, *Eae3*, *Eae26*, *Eae27*) have been identified in crosses between the B10.RIII and RIIS/J strains^{34, 65}. The B10.RIII strain is susceptible to induction of chronic relapsing EAE upon immunization with the MBP peptide 89-101²⁹. In humans, the HLA-DR2 MHC haplotype is associated with susceptibility to MS²⁰. The MBP peptide 84-102 has been shown to bind HLA-DR2, demonstrating that this peptide may be important for MS pathogenesis^{74, 75}. This was confirmed in studies of a humanized mouse model where HLA-DR2 was expressed and the transgenic mice developed EAE upon challenge with the MBP peptide⁴³.

In EAE models used for genetic studies of disease development, the respective parental strains express the same MHC haplotype (MHC congenics), whereas the rest of the genetic background is diverse. Since one of the parental strains is resistant to disease, the aim of the experiments is to find genes outside the MHC important for the development of the disease. The B10.RIII strain obtained its MHC region, H-2^f, from the EAE-resistant strain RIIS/J. Except for the MHC and a large region on chromosome 10, the two strains have different genetic backgrounds.

To date, 27 non-MHC *Eae* loci have been reported (<http://www.informatics.jax.org> and³⁴) (Table 1). The *Eae* loci are linked to different traits of disease. Interestingly, the linkages are to a large extent sex specific, meaning that a certain trait of the disease is linked to a particular genetic location only in one of the sexes. An important issue is to investigate if the differences between male and female mice can explain the sex difference in susceptibility to autoimmune disease in humans. Moreover, in crosses between the SJL/J/B10.S/DvTe and the B10.RIII/RIIS/J strains, several clinical subtypes have been

Table 1. EAE loci in mouse models

Eae locus	Location		Sex specificity	Trait	Reference
	Chr ¹	cM ²			
<i>Eae1</i>	17	20		incidence	17
<i>Eae2</i>	15	15		incidence	65
<i>Eae3</i>	3	42		incidence	65
<i>Eae4</i>	7	50		incidence, spinal chord histopathology	9
<i>Eae5</i>	17	21		incidence	11
<i>Eae6a</i>	11	7		severity	11
<i>Eae6b</i>	11	24		duration	11
<i>Eae7</i>	11	48		severity, acute disease	9, 11
<i>Eae8</i>	2	103		incidence, severity	11
<i>Eae9</i>	9	35		duration	11
<i>Eae10</i>	3	72		onset	11
<i>Eae11</i>	16	41	male	incidence, brain histopathology	9
<i>Eae12</i>	7	12	female	remitting relapsing disease	9
<i>Eae13</i>	13	37	male	monophasic disease	9
<i>Eae14</i>	8	21	female	incidence	6, 15
<i>Eae15</i>	10	16	male	brain histopathology	10
<i>Eae16</i>	12	6		spinal chord histopathology	10
<i>Eae17</i>	10	36	female	severity, spinal chord demyelination	6
<i>Eae18</i>	18	syntenic		incidence	3
<i>Eae19</i>	19	34	male	brain demyelination	10
<i>Eae20</i>	3	14		spinal chord demyelination	10
<i>Eae21</i>	2	37	female	brain histopathology	10
<i>Eae22</i>	11	61	female	brain histopathology	10
<i>Eae23</i>	11	38	male	spinal chord histopathology	10
<i>Eae24</i>	8	10		CNS inflammation	15
<i>Eae25</i>	18	54	male	spinal chord histopathology	6
<i>Eae26</i>	5	57	male	acute disease	34
<i>Eae27</i>	1	82	female	remitting relapsing disease	34

¹ Chromosome.

² cM position of genetic marker correlated to linkage peak according to <http://www.jax.org> or reference.

reported, suggesting that the disease course is to a large extent dependent on the genetic context^{9, 34}. The finding that different subtypes of disease are significantly linked to different genetic loci is important for understanding the variation in disease course observed in MS patients¹³.

Two large genetic linkage studies have been performed in crosses between the B10.RIII and RIIIS/J strains. In a (B10.RIII × RIIIS/J)F2 intercross, two main loci were identified: *Eae2* on chromosome 15 and *Eae3* on chromosome 3⁶⁵. Interestingly, it was reported that the two loci have an additive effect on the incidence of EAE. The second study included 402 offspring from a (B10.RIII × RIIIS/J)F1 × B10.RIII back-cross³⁴. This study revealed 6 genetic regions linked to different traits of EAE. Two of the QTL, in chromosomes 1 (*Eae27*) and 5 (*Eae26*), had not previously been reported in mouse EAE. The other QTL identified in this experiment overlapped with previously reported *Eae* loci. One could speculate that the *Eae* loci that are shared between the EAE models control common pathways in the disease process, whereas the loci that are unique for each model might reflect the different induction protocols. These differences might also depend on experimental set up, i.e. differences in numbers of animals and phenotyping protocols.

Mouse chromosome 11

Five *Eae* loci are located on chromosome 11. *Eae6b*, *Eae23*, and *Eae7*, on the central part of the chromosome, are linked to duration of disease (*Eae6b*), spinal chord lesions in male mice (*Eae23*), and severity, duration, and development of monophasic EAE (*Eae7*)^{10, 11, 66}. The loci, which are located within a rather limited distance, were initially described in the model including the SJL/J/B10.S/DvTe strains, but recently the same region on chromosome 11 was linked to incidence of chronic disease, day of onset, severity, and duration of disease in a back-cross between B10.RIII and RIIIS/J³⁴. A number of genes of interest for activity in the immune system are located on chromosome 11. Among those are the genes for interleukin (IL)-3, IL-4, IL-5, IL-13, granulocyte-macrophage colony-stimulating factor (GM-CSF), iNOS (inducible nitric oxide synthase 2), and genes coding for different chemokines. The genes for the chemokines MCP-1 (mouse chemokine monocyte chemotactic protein-1) and MCP-5 are located on central chromosome 11. In a recently reported analysis of gene expression in spinal chord from transgenic mice developing spontaneous EAE, MCP-1 and MCP-5 were differently expressed in tissue from mice with preclinical and clinical EAE⁴⁷. Interestingly,

Teuscher et al.⁶⁶ reported sequence variations in the genes for MCP-1 and MCP-5 between the susceptible SJL/J and the EAE-resistant B10.S/DvTe mouse strains. In mice with MOG-induced EAE, the differential expression of genes at different times during the disease course was investigated. In this experiment the MCP-1 and MCP-5 genes were not differentially expressed²⁵. Different mouse strains and protocols were used in the studies mentioned and this could explain the different results. In addition, it is important to note that polymorphisms in structural genes do not always result in differential expression of the gene.

A number of QTL for disease development in other models of inflammatory disease have been mapped to the central region on chromosome 11: *Lbw8* (lupus)⁷⁷, *Pgia7* (proteoglycan-induced arthritis)⁵⁴, *Orch3* (orchitis)⁴⁸, *Idd4* (insulin-dependent diabetes)⁶⁸, *Bbaa4* (*Borrelia burgdorferi*-associated arthritis)⁷², and *Sle13* (lupus)⁵¹. In the rat, the region homologous to the middle part of chromosome 11 in the mouse resides on chromosome 10 and has been linked to development of EAE⁵⁸. In addition, a region further telomeric on rat chromosome 10 has been linked to MOG-induced EAE²⁸. Interestingly, the syntenic chromosomal location in humans has been associated to susceptibility to MS⁵⁹. However, none of the currently defined *Eae* loci on mouse chromosome 11 seem to cover this region. A recent genetic linkage study on RA patients showed an association to a polymorphism in the gene encoding solute carrier family 22 member 4 (*SLC22A4*) on chromosome 5q31⁶⁹. This genetic location is homologous to the central portion of mouse chromosome 11, which has been linked to disease development in experimental model of arthritis^{54, 72}.

Mouse chromosome 16

Eae11 on chromosome 16 was first identified in a cross between the SJL/J and B10.S/DeTv strains and was linked to incidence of EAE in male mice⁹. In a back-cross between the B10.RIII and RIIIS/J strains, linkage to the day of disease onset in male mice was found close to *Eae11*³⁴. The *Cbl-b* gene, coding for a ubiquitin-protein ligase, has been suggested as a susceptibility gene for diabetes in a rat model for type I diabetes⁷⁶. This gene is located in a region on mouse chromosome 16, linked to EAE development in the two mouse models. In addition, this region harbors the genes for CD200, the CD200 receptor, and CD47, which are important for the communication between different cell-types and macrophages⁴. Other interesting QTL on chromosome 16 are *Aod1*, important for susceptibility to

autoimmune ovarian dysgenesis, which develops as a result of thymectomy at day 3 after birth⁷¹, and *Pgia10*, controlling proteoglycan-induced arthritis⁵⁴.

Mouse chromosome 5

On chromosome 5 we recently found a novel QTL linked to the development of remitting EAE in male mice (*Eae26*)³⁴. No *Eae* loci have previously been described on this chromosome, but the actual region contains two QTL for *Borrelia burgdorferi*-induced arthritis in mice⁷². The gene for osteopontin is found in this area, and in two different studies a role for osteopontin in the development of EAE was reported^{12, 30}. Furthermore, osteopontin gene transcripts were found to be up-regulated in MS lesions¹². The gene for this pro-inflammatory cytokine is located in, or close to, the *Eae26* region in the mouse. In mice made deficient in osteopontin the investigators observed a significant decrease in severity of EAE¹². In another study, however, no effect on disease outcome was observed in EAE, collagen-induced arthritis, and anti-collagen type II antibody-induced arthritis⁷. Importantly, these reports have given rise to discussion and concern about polymorphic genes derived from the 129 mouse strain adjacent to the “knocked out” gene, which could be the actual genes controlling the observed differences in disease outcome.

Another gene in this area is the *Ncf1*, which codes for neutrophil cytosolic factor 1, a constituent of the NADPH oxidase complex. Recently, by positional cloning the *Ncf1* gene was demonstrated to be a susceptibility gene for pristane-induced arthritis in rats⁵³. In the mouse, this gene is located close to the *Eae26* region on chromosome 5.

Mouse chromosomes 1 and 6

The telomeric part of mouse chromosome 1 harbors a large number of QTL for disease phenotypes in several models of autoimmune diseases: CIA (*Cia9*)³³, SLE (*Sle1*, *1a*, *1b*, *1c*; *Sle9*)^{22, 50}, serum transfer-induced arthritis (*Stia1*)³¹, and experimental autoimmune orchitis (EAO; *Orch4*)⁴⁸. A locus linked to remitting EAE in female mice was identified in this region in the B10.RIII/RIIS/J model (*Eae27*)³⁴. Further fine-mapping and subsequent identification of the genes behind the QTL will reveal whether this reflects genetic elements that control common pathways for different inflammatory models.

One such locus important for susceptibility to both EAE and EAO is *Bphs1* on mouse chromosome 6, which controls histamine sensitization⁶⁴. Recently,

the gene behind this locus was demonstrated to be the histamine H₁ receptor⁴².

Genes for myelin proteins in EAE QTL

The end of chromosome 18 has been linked to incidence of disease in studies of EAE in mouse (*Eae18*, *Eae25*)^{3, 6} and rat crosses⁵. This region includes the gene for MBP, which has been indicated as a candidate gene in MS^{19, 56, 67}. The gene for MOG is located close to the MHC region on mouse chromosome 17. Oligodendrocyte-myelin glycoprotein is located in the middle part of chromosome 11 and, as discussed above, a number of putative genes controlling development of EAE exist at this region. In a study of gene expression in MS lesions, the oligodendrocyte-myelin glycoprotein gene was found to be differentially expressed when samples from lesions of varying activation stages were compared⁴¹. Lymphocytes reactive to myelin proteins are present in healthy individuals and are normally kept in a state of tolerance^{1, 44}. By immunization with myelin antigens in rodent models, the cells are triggered and mount a pathological immune response towards these antigens in susceptible mouse strains. However, it remains to be shown if differential expression of myelin proteins/peptides during the autoimmune phase in the brain or during negative selection of autoreactive T cells in the thymus would contribute to MS/EAE pathogenesis.

BEYOND THE GENETIC LINKAGE ANALYSES

Once the initial linkage analysis is finalized, the existence of a gene or genes within the QTL that control a specific trait of EAE needs to be confirmed and then the putative gene or genes can be identified. The genetic linkage analyses, depending on the number of animals, normally leave the investigator with a linkage peak comprising 20–30 cM, consisting of several hundred genes. In order to come closer to the actual susceptibility gene(s), the interval linked to the trait needs to be considerably smaller. One way to continue the search for a small genetic region that still controls the trait is to establish congenic mouse lines⁷⁰. The interesting genetic region from one of the parental strains is bred onto the background of the other parental strain. By further intercrossing the congenic mice, recombinations within the fragment will create mice with even smaller congenic fragments. Analyzing the congenic mice for the important phenotype should reveal whether the trait is controlled by a gene, or genes, within the fragment or not. When a congenic region controlling a specific trait contains only a few genes, the candidates can be investigated for sequence polymorphisms and differential expression in relevant tissues.

Although a congenic interval contains the correct genetic element(s) needed to contribute to the expression of a particular trait, the expected phenotype might not be observed in the congenic animals. One could speculate that this is due to the fact that the background of the congenic mouse does not make up the correct genetic context for the important gene(s), i.e. genetic interactions are needed for complete penetrance of the gene. One example of this is in an F2 intercross between the B10.RIII (susceptible) and RIIIS/J (resistant) strains, where homozygosity for RIIIS/J genes on the centromeric part of chromosome 15 (*Eae2*) protected the animals from developing EAE⁶⁵. Subsequently, *Eae2* congenic mice were produced and tested for EAE. The results showed that the mice carrying RIIIS/J genes, in contrast to what was expected, developed EAE to the same extent as the controls³². To test the hypothesis that other genes might be needed to interact with *Eae2* in order to obtain the expected phenotype, 259 animals from a gene-segregating cross between *Eae2* congenic mice (*Eae2* from the RIIIS/J on the B10.RIII background) and RIIIS/J were tested for EAE. With the *Eae2* region fixed for RIIIS/J genes, one significant linkage for susceptibility to EAE was found on chromosome 7, close to the *Eae4* locus. *Eae4* was previously identified in a cross between SJL/J and B10.S/DvTe and linked to the incidence of EAE⁹. It remains to be investigated if disease development will be altered in mice double-congenic for *Eae2* and the region on chromosome 7.

It is becoming increasingly clear from the genetic studies of complex disease models, which involve many pathways, that there are gene interactions and that these will further complicate analyses of the genes involved in a disease. In addition, there are QTL that have been split up into several smaller regions, suggesting that many genes contributing to a particular phenotype are present in a gene cluster⁵⁰. If this is the case, it will be necessary to separate the smaller QTL and subsequently the underlying genes

in order to decide which one is contributing to disease and how. There might also occur interactions between the genes within the cluster. In order to dissect two genetic regions suspected to contain more than one important gene in each region, we have recently intercrossed *Eae2* and *Eae3* congenic mice (manuscript in preparation). It was previously suggested that the two QTL interact to cause disease⁶⁵. The strategy has been to breed a “partially advanced intercross”, that is, mice from the first intercross were randomly picked for the next generation, and this was continued for up to 8 generations. Using this method we have obtained mice with a high density of recombinations and, consequently, increased the possibility to derive small congenic fragments within each region. By studying the mice with recombinations in various combinations of *Eae2* and *Eae3*, we can conclude that the two QTL contain several genes of importance for the development of inflammatory disease, each within a small-defined region. By using small fragments in the right genetic combination that results in the strongest phenotype, it will be possible to find individual candidate genes even in genetically complex regions like *Eae2* and *Eae3*.

Extensive work has to be done before we know what natural variants of different genes make up the background for susceptibility to a chronic inflammatory disease like MS. Studies of arthritis in a rat model have, however, already revealed the possibility to positionally clone a gene controlling the development of a complex inflammatory disease by the disease-to-gene strategy⁵³. The knowledge of biological pathways important for susceptibility to autoimmune disease will make it possible to understand how to develop new therapies.

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Added in proof:

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