Combined-cross analysis of genome-wide linkage scans for experimental autoimmune encephalomyelitis in rat

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Abstract

Unbiased genetic analysis of experimental autoimmune encephalomyelitis (EAE) can provide insights into the pathogenesis of multiple sclerosis. To date five genome-wide scans using F2 crosses between different inbred rats have been performed with the aim of defining EAE-regulating quantitative trait loci (QTLs) as the starting point for identification of the underlying genes. We here report the first combined-cross analysis of three F2 crosses previously performed in our group. The majority of QTLs was shared between the different strain combinations and was therefore reproduced by the combined-cross analysis. Consequently, combined-cross analysis improved the power to detect QTLs with modest effects and narrowed QTL confidence intervals. The findings also demonstrate a lack of power in previous F2 crosses and encourage future use of larger populations. Moreover, the allelic states of shared QTLs could be established, thus providing critical information for narrowing QTLs and identifying the key polymorphism by subsequent haplotype analysis.

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Unbiased identification of genes that regulate disease is a powerful phenotype-driven approach to define primary disease mechanisms, which in turn may lead to the design of more rational and effective therapeutic strategies and/or prevention. Our interest is in the field of multiple sclerosis (MS). In this disease, linkage analyses in large numbers of families have failed to reveal any significant gene region apart from the HLA, as recently demonstrated in a SNP-based whole genome scan [1]. We have concentrated our genetic analyses on chronic forms of experimental autoimmune encephalomyelitis (EAE) under the assumption that gene polymorphisms critically involved in this experimental disease can yield mechanisms relevant and accessible for drug targeting also for humans. It may furthermore identify candidate genes to be studied by more powerful association studies in large cohorts of MS cases and controls. With these aims genetic analyses of EAE were initiated in the 1990s [2–8]. Both MS and EAE are complex diseases determined by multiple genetic and environmental factors and interplay between them [9,10]. Genome-wide scans have been utilized in an attempt to identify multiple regions simultaneously, called quantitative trait loci (QTLs), that regulate different EAE phenotypes [7,8,11–18]. The effects of several of these QTLs have subsequently been confirmed in congenic strains and are now being subjected to positional cloning [19–25].

A few successful demonstrations of positional cloning such as CD36 in hypertension [26], Ncf-1 in arthritis [27], CTLA-4 in type 1 diabetes [28], and Fcgr3 in glomerulonephritis [29] demonstrate that it is possible to identify genes underlying QTLs. This endeavor has, however, proven to be considerably more challenging than what was expected from experience with monogenic diseases. The reasons lie in the intrinsic complexity of the disease, primarily the modest effect of each susceptibility gene, the incomplete genetic penetrance, clustering of susceptibility genes, and complex gene–gene and gene–environment interactions [24,30]. Genome-wide analyses in F2 crosses using a number of different inbred rat strains aimed (i) at addressing the issue of genetic heterogeneity and detection of additional QTLs that will operate exclusively in
certain strain combinations, but also (ii) at providing reproduction and therefore confirmation of QTLs and reassure likelihood of positional cloning and finally (iii) at identifying common QTLs that segregate in different strain combinations, which could give a basis for subsequent gene identification with the aid of haplotype analysis. Although powerful, genome-wide analyses depend on factors such as heritability, size of F2 population, diversity between parental inbred strains, and density of genetic markers, which are difficult to predict a priori [31]. Consequently, F2 genome-wide scans in EAE identified a number of QTLs that displayed modest effects and many of them could not be reproduced with certainty [6–8,17,32].

Ultimately, an efficient way to achieve these aims is to combine the raw data obtained in the different crosses and perform a combined-cross analysis. Whereas such and similar analyses have been reported for MS [33–35], none has yet been published for EAE. Recently, Li and colleagues published analytical methods and their application of combined-cross analysis of QTLs that regulate cholesterol levels in inbred mice [36]. With the rat genome sequences completed, statistical tools for QTL analysis developed, and data from previous F2 crosses, we can now investigate the architecture of QTLs that regulate rat autoimmune neuroinflammation in more detail by performing combined-cross analysis. We here present the outcome of combined-cross analysis on pooled data from three different F2 crosses [7,8,17] that we hope will motivate future comprehensive efforts.

**Results**

**Genome-wide analysis**

We performed the analysis on combined data from three F2 crosses (Table 1) using affection status of rats as EAE phenotype and the F2 cross as a covariate. Detailed lod score plots and values are given in Fig. 1 and Table 2. Eight QTLs that regulate susceptibility to EAE in rats were detected in the three F2 crosses. Four significant QTLs (on chromosomes 1, 9, 13, and 17) and one suggestive QTL (on chromosome 15) were detected when the cross covariate was analyzed as interactive. Two significant (on chromosomes 1 and 9) and five suggestive (on chromosomes 12, 14, 15, 17, and X) QTLs were detected when the cross covariate was analyzed as additive. It is interesting to note that most of the QTLs (on

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**Table 1**

<table>
<thead>
<tr>
<th>Cross</th>
<th>Type</th>
<th>Susceptible</th>
<th>Resistant</th>
<th>EAE %</th>
<th>N&lt;sub&gt;a&lt;/sub&gt;</th>
<th>N&lt;sub&gt;b&lt;/sub&gt;</th>
<th>Type&lt;sub&gt;d&lt;/sub&gt;</th>
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<tr>
<td>1</td>
<td>F2</td>
<td>DA</td>
<td>ACI</td>
<td>MOG</td>
<td>19</td>
<td>188</td>
<td>Extreme</td>
</tr>
<tr>
<td>2</td>
<td>F2</td>
<td>DA</td>
<td>BN</td>
<td>WSC</td>
<td>39</td>
<td>285</td>
<td>Extreme</td>
</tr>
<tr>
<td>3</td>
<td>F2</td>
<td>LEW.AV1</td>
<td>PVG.AV1</td>
<td>MOG</td>
<td>71</td>
<td>185</td>
<td>Whole</td>
</tr>
</tbody>
</table>

<sup>a</sup> EAE was induced by immunization with recombinant MOG (1-125 aa) in IFA (MOG) and whole spinal cord homogenate in CFA (WSC).

<sup>b</sup> Size of F2 population that was subjected to EAE induction.

<sup>c</sup> Number of microsatellite markers that was used to genotype the genome.

<sup>d</sup> F2 rats that were selected for genotyping were either rats that displayed extreme phenotypes (extreme) or a whole F2 population (whole).

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**Fig. 1.** QTLs that regulate susceptibility to EAE detected in individual F2 crosses and after combined-cross analysis. The lod scores generated by the multiple imputation method are given on the y axis and chromosomal positions are given on the x axis (small vertical lines represent positions of genotyped microsatellite markers). Shared QTLs were detected using cross as an additive covariate (gray line), whereas cross-specific QTLs were detected using cross as an interactive covariate (black line). Sharing is indicated by the lack of a significant difference between lod scores obtained using the cross as interactive and additive covariate (lod score difference <2.1). Horizontal solid and dashed lines indicate 0.05 and 0.63 significance thresholds, respectively, generated with 1000 permutations. The 0.05 levels for significant linkage were 4.1 (ALL, F2 cross index as interactive covariate), 3.1 (ALL, F2 cross index as additive covariate), 3.0 (DA × ACI), 3.1 (DA × BN), and 3.5 (LEW × PVG).
Table 2
Summary of QTLs that regulate susceptibility to EAE detected in individual F2 crosses and after combined-cross analysis

| Chr | Single F2 crosses | Combined-cross analysis | LOD_
<table>
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<tbody>
<tr>
<td></td>
<td>DA × ACI</td>
<td>DA × BN</td>
<td>LEW × PVG</td>
</tr>
<tr>
<td>1</td>
<td>1.8+</td>
<td>3.0**</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>5.4†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>2.3**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>3.5†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1.9**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>1.3*</td>
<td>10–100 (90)</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>1.6*†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>2.3**</td>
<td></td>
<td></td>
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</table>

- ** Values represent lod scores, with 95% confidence intervals for QTL location in megabases, generated by bootstrapping, given below the lod score in italic (the size of the 95% confidence interval is indicated in parentheses). Significance thresholds were generated with 1000 permutations (**, *, and ns indicate 0.63 cutoff, just below 0.63 cutoff, and nonsignificant values, respectively).
- Significance thresholds were generated with 1000 permutations (+, †, and ‡ indicate significant, suggestive, and supportive QTL in the original F2 analysis).
- Combined-cross analysis performed using F2 cross index as interactive covariate (LODint).
- Combined-cross analysis performed using F2 cross index as additive covariate (LODadd).
- Significance of difference between lod scores generated using F2 cross index as interactive versus additive covariate (LODint vs LODadd). Significant values, p < 0.05, are for LOD difference >2.1.
- Difference between lod scores generated using single- and two-QTL model (difference in lod scores >4.5 is considered to be p < 0.05).

EAE QTL on rat chromosome 1

The QTL on rat chromosome 1 displayed LODint and LODadd of 4.9 and 3.7, respectively, in the vicinity of marker D1Rat4 at 12 Mb (Fig. 2A). This QTL had previously demonstrated evidence supporting linkage and suggestive linkage in the (DA × BN)F2 cross and (LEW × PVG)F2, respectively (Table 2). Lack of significant difference between LODint and LODadd (p > 0.5) indicates that this QTL is shared between different F2 crosses (Table 2). This notion is further supported by a dramatic reduction in the 95% confidence interval from 100–110 to 16–23 Mb after combined-cross analysis (Fig. 2A, Table 2). The DA/LEW strains share the allelic variant of the underlying gene that predisposes for EAE, whereas the ACI/BN/PVG strains share the allelic variant that protects from EAE (Fig. 3A).

Eae4 on rat chromosome 9

The QTL on the centromeric end of rat chromosome 9, previously designated Eae4, was detected with the maximum LODint and LODadd of 5.6 and 5.4, respectively, for marker D9Rat40 at 9 Mb (Fig. 2B). Eae4 displayed significant linkage in the (DA × BN)F2 cross, whereas there was no evidence for linkage to EAE in the other two crosses, (DA × ACI)F2 and (LEW × PVG)F2 (Table 2). Interestingly, there was no difference between LODint and LODadd suggesting that Eae4 is a shared QTL (Table 2). Closer inspection of the influence of the peak marker on EAE in the (DA × BN)F2 cross as well as markers with similar positions in two other F2 crosses suggests an influence of genotype on phenotype that did not reach statistical significance (Fig. 3B). We could therefore determine the allelic state of the gene underlying Eae4 as follows: DA/LEW as disease-promoting and ACI/BN/PVG as disease-protecting. Furthermore, combined-cross analysis narrowed the 95% confidence interval from 62 Mb in the original cross to 47 Mb (Fig. 2B, Table 2).

Eae17—transgressive QTL on rat chromosome 13

One of the strongest QTLs was detected on rat chromosome 13 with LODint of 5.3 for marker D13Rat23 at 47 Mb. This QTL, designated Eae17, has previously been detected in the (DA × ACI)F2 and the (LEW × PVG)F2 crosses (Table 2). Interestingly, analysis using cross as an additive covariate resulted in LODadd of 0.7, with a significant difference between

chromosomes 1, 9, 12, 14, 15, 17, and X) were shared between all three crosses or subset of crosses although most of them were not identified after each individual F2 cross analysis (Fig. 1). This sharing is indicated by the lack of significant difference between lod scores obtained using the cross as interactive and additive covariate, further in the text referred to as LODint and LODadd, respectively. The most significant QTLs were analyzed in detail.

EAE QTL on rat chromosome 1

The QTL on rat chromosome 1 displayed LODint and LODadd of 4.9 and 3.7, respectively, in the vicinity of marker D1Rat4 at 12 Mb (Fig. 2A). This QTL had previously demonstrated evidence supporting linkage and suggestive linkage in the (DA × BN)F2 cross and (LEW × PVG)F2, respectively (Table 2). Lack of significant difference between LODint and LODadd (p > 0.5) indicates that this QTL is shared between different F2 crosses (Table 2). This notion is further supported by a dramatic reduction in the 95% confidence interval from 100–110 to 16–23 Mb after combined-cross analysis (Fig. 2A, Table 2). The DA/LEW strains share the allelic variant of the underlying gene that predisposes for EAE, whereas the ACI/BN/PVG strains share the allelic variant that protects from EAE (Fig. 3A).

There is a possibility of an additional QTL on rat chromosome 1, located around 100–120 Mb (Fig. 2A), that would be specific for the LEW/PVG cross. However, our data did not have sufficient power to resolve this QTL.
LOD\text{int} and LOD\text{add}, i.e., two models ($p<0.0003$). This could indicate that Eae17 is a cross-specific QTL. However, closer inspection of allelic effects in the original (DA×ACI)F2 cross revealed that this is an example of a transgressive QTL in the DA/ACI strain combination, where an allele from the resistant ACI strain predisposes for the disease (Fig. 3C). We recoded alleles according to the effect on susceptibility and reanalyzed the data (Fig. 4A). The difference between LOD\text{int} of 4.4 and LOD\text{add} of 1.2 was still significant ($p<0.005$). This could indicate that there are two QTLs in the Eae17 region, one that segregates in the DA/ACI strain combination with the ACI allele being disease predisposing and another that segregates in the LEW/PVG strain combination with the LEW allele being disease predisposing. We performed analysis on the combined data from (DA×ACI)F2 and (LEW×PVG)F2 crosses, omitting the (DA×BN)F2 cross (Fig. 4B). There was still a significant difference between LOD\text{int} and LOD\text{add} of 4 ($p<0.001$). Furthermore, the possibility of linked QTLs in the region, investigated by implementing a pair-scan analysis, resulted in the lod score of 11.3 for the existence of two QTLs at positions 38 and 48 Mb. The difference between this lod score and LOD\text{int} is 4.6, which is just above the significance level of 4.5. We might then conclude that there are two linked QTLs, one that operates in DA/ACI and the other in LEW/PVG combinations. This is further supported by the lack of reduction in the 95% confidence interval (Table 2). However, we have to be cautious in the interpretation, considering the allelic effect of this region, ACI recessive, LEW dominant, and a tendency for DA/BN heterozygous in the DA/ACI, LEW/PVG, and DA/BN strain combination, respectively (Fig. 3C), and that our allele coding in the combined-cross analysis cannot distinguish different heterozygotes.

Eae19 on rat chromosome 15

Evidence for a QTL on rat chromosome 15 was detected with LOD\text{int} and LOD\text{add} of 3.2 and 2.6, respectively, for marker D15Rat71 at 85 Mb (Fig. 2D). Evidence supporting this QTL, designated Eae19, although not significant, came from all three F2 crosses (Table 2). The DA/LEW rats share the allelic variant of the underlying gene that predisposes for EAE, whereas ACI/BN/PVG share the allelic variant that protects from EAE (Fig. 3D). There was however no reduction in 95% confidence intervals, probably due to the modest effect of Eae19 (Fig. 2D, Table 2).

Eae19 accounts for approximately 1–3% of phenotypic variance depending on the F2 cross, representing a modest QTL.

Fig. 2. Significant and suggestive QTLs detected by combined-cross analysis of three F2 crosses. The lod scores generated by the multiple imputation method are given on the y axis and chromosomal positions on the x axis. Shared QTLs were detected using cross as additive covariate (ALL ADD), whereas cross-specific QTLs were detected using cross as interactive covariate (ALL INT). Sharing is indicated by the lack of a significant difference between lod scores obtained using the cross as interactive and additive covariate (lod score difference <2.1). Horizontal dashed lines indicate significant and suggestive linkage at levels of 0.05 and 0.63, respectively, generated with 1000 permutations. The 95% confidence intervals, generated by bootstrapping, are represented by horizontal gray bars.
as the majority of EAE QTLs. Simulation of F2 populations of different sizes generated lod scores for \textit{Eae19} of 1.4 ± 0.5, 1.6 ± 0.9, 2.4 ± 0.3, 3.2 ± 0.3, and 4.2 ± 0.9 in data sets comprising 200, 400, 600, 800, and 1000 F2 rats, respectively. The threshold values for significance, determined by permutation analysis, were \textit{LOD} ≥ 3.2, \textit{LOD} ≥ 2.9, and \textit{LOD} ≥ 1.9 for 0.05, 0.1, and 0.63 significance levels, respectively. In populations of 200 and 400 F2 rats, covering the most commonly utilized population size span, lod scores for \textit{Eae19} were below any evidence of linkage. In the population of 600 F2 rats \textit{Eae19} lod scores display some evidence of linkage but still below 0.1 levels. This modest QTL displayed significant linkage in populations of 800 and 1000 F2 rats.

\textbf{EAE QTL on rat chromosome 17}

The QTL on rat chromosome 17 displayed significant \textit{LOD}_{int} and suggestive \textit{LOD}_{add} of 4.3 and 2.6, respectively, in the vicinity of the marker D17Rat67 at 35 Mb (Fig. 2E). Evidence for this QTL was detected in the previous (DA × BN) F2 and (LEW × PVG) F2 crosses (Table 2). The difference between \textit{LOD}_{int} and \textit{LOD}_{add}, although not statistically significant \((p=0.1)\), might indicate that this QTL does not segregate in the DA/ACI strain combination. Therefore, DA/LEW share the allelic variant of the underlying gene that predisposes for EAE at 35 Mb, whereas BN/PVG share the allelic form that protects from EAE (Fig. 3E). Furthermore, a shape of the lod curve detected in the (LEW × PVG) F2 cross indicates a possibility of additional QTL around 65–75 Mb in this strain combination (Fig. 2E).

The QTL on rat chromosome 17 displayed significant \textit{LOD}_{int} and suggestive \textit{LOD}_{add} of 4.3 and 2.6, respectively, in the vicinity of the marker D17Rat67 at 35 Mb (Fig. 2E). Evidence for this QTL was detected in the previous (DA × BN) F2 and (LEW × PVG) F2 crosses (Table 2). The difference between \textit{LOD}_{int} and \textit{LOD}_{add}, although not statistically significant \((p=0.1)\), might indicate that this QTL does not segregate in the DA/ACI strain combination. Therefore, DA/LEW share the allelic variant of the underlying gene that predisposes for EAE at 35 Mb, whereas BN/PVG share the allelic form that protects from EAE (Fig. 3E). Furthermore, a shape of the lod curve detected in the (LEW × PVG) F2 cross indicates a possibility of additional QTL around 65–75 Mb in this strain combination (Fig. 2E).

Additional QTLs have been detected on chromosomes 12, 14, and X that have not been described in detail (Fig. 1, Table 2). Some indications of influences from chromosomes 5, 10, and 19 that did not reach levels of suggestive linkage were detected. However, a region on chromosome 5 (\textit{LOD}_{int} = 1.75) was detected at the same position as \textit{Eaex} in the (E3 × DA) F2 cross [32]. This QTL displays the following allele distribution: LEW/BN vs DA/PVG. Therefore the allele from the susceptible DA strain confers protection from disease, which
is in accordance with the effect detected in the DA/E3 strain combination [32].

Discussion

Combined-cross analysis of three F2 crosses in EAE, which involved five inbred rat strains, resulted in the following main conclusions: (i) many QTLs are shared between strains, that is, they appear in different strain combinations, and are thus reproduced by the combined-cross analysis; (ii) some QTLs are specific for a certain cross, that is, they appear only in a specific strain combination, reflecting genetic heterogeneity; and (iii) combined-cross analysis enables the determination of the allelic state of the QTL in particular strains and, in the case of shared QTLs, reduces confidence intervals.

Eight QTLs that regulate susceptibility to EAE were identified by combined-cross analysis. There were additional influences from regions on chromosomes 5, 10, and 19 that did not reach levels suggestive of linkage. Additional QTLs would certainly have appeared if other EAE phenotypes, in particular severity and chronicity, were analyzed. However, these phenotypes are more difficult to standardize between the three F2 crosses and we have, therefore, chosen to run our analysis on a more robust phenotype, such as EAE affection status.

We conclude that many shared QTLs were detected in the combined-cross analysis, which had not reached significance in each individual F2 cross. We believe that a main reason for this is the number of individuals analyzed, which affects power. Thus, the size of a single F2 population of 200–300 rats, commonly used in analyses of EAE, is apparently not sufficient to reproducibly detect multiple QTLs with modest effects such as those that regulate EAE. An illustrative example is Eae19 on chromosome 15, which was here detected as a suggestive QTL in the combined cross comprising 658 F2 rats and previously displayed only weak evidence for linkage in individual F2 crosses [7,8,17]. The influence of this QTL has independently been confirmed by introducing the ACI allele on the DA background in a congenic strain as well as in the DA/PVG intercross [25], thus confirming the combined-cross analysis data. A population size that allows detection of significant linkage to Eae19, representing a true QTL of a modest effect, is approaching 800 F2 rats. Detection of the majority of EAE QTLs, which display influences similar to those of Eae19, will therefore require populations of a minimum of 600 F2 rats, with significant linkages occurring in 800 to 1000 F2 rats. Combined-cross analysis may also detect new QTLs not apparent in individual F2 crosses. Thus in the present analysis, the regions on chromosomes 5 and 19 appeared with some evidence of linkage, not at all apparent in our previous three crosses. The relevance of these particular QTLs is supported by their demonstration in an independent (E3 × DA)F2 cross [32].

A further inherent complication in the analysis of different strain combinations relates to genetic heterogeneity. Thus, there might be additional QTLs on chromosomes 1 and 17 that would segregate only in the LEW/PVG combination, reflecting genetic heterogeneity, which is seen in human MS as well [37]. Consequently, these regions did not reach statistical significance in the present combined-cross analysis. Together, these findings strongly advise utilization of larger F2 crosses to investigate genetic heterogeneity and to reproduce previously identified QTLs. Importantly, shared QTLs indeed found in combined-cross analysis demonstrate a crucial confirmation, which in turn motivates further positional cloning attempts. Meta-analysis performed on several complex human diseases suggested a similar selection of the most promising QTLs for further analysis [38].

There are a number of reasons, in addition to F2 population size and analyzed phenotype, responsible for the failure of individual F2 crosses to detect certain QTLs. The genomic coverage might explain the case of shared QTL on chromosome 1 at 12 Mb that was detected in the (DA × BN) F2 and the (LEW × PVG)F2 crosses [7,17]. The most probable reason for failure of the (DA × ACI)F2 cross to detect this QTL is the lack of marker coverage in this region (first marker is at 41 Mb) [8]. Investigation of the effects of genotypes at the first genotyped marker in (DA × ACI)F2 suggests a weak effect that might reflect even stronger upstream effects, which unfortunately could not be determined due to the lack of genotyped markers in the region (Fig. 3A). The differences in EAE induction protocols could be the explanation for failure of the (DA × ACI)F2 and (LEW × PVG)F2 crosses to detect Eae4 on rat chromosome 9, the strongest QTL detected in the (DA × BN)F2 cross [7]. The first two crosses were performed in EAE induced with recombinant MOG in incomplete Freund’s adjuvant (IFA), whereas later, EAE was induced with whole spinal cord homogenate in complete Freund’s adjuvant. Recombinant MOG/IFA induction might primarily involve other regions, leaving just a small proportion of influence to Eae4. This is supported by the influence of genotypes on phenotype that did not reach statistical significance (Fig. 3B). A similar effect has been demonstrated for pertussis toxin in murine EAE [15]. Nevertheless, a possibility of an additional linked gene or the third allelic variant can not be excluded. Particularly interesting is the QTL on chromosome 13, which displayed linkage in the (DA × ACI)F2 and (LEW × PVG)F2 crosses [8,17], and combined-cross analysis suggested that it might contain two linked cross-specific QTLs. The first QTL, around 40 Mb, segregated in the DA/ACI combination and represents an example of a transgressive QTL, in which the allele from the resistant ACI strain predisposes for disease in a recessive manner. The second QTL, Eae17, around 50 Mb, segregated in the LEW/PVG combination with the LEW allele predisposing for disease in a dominant manner. However, we have to be cautious in the interpretation of the data since there are several arguments suggesting that Eae17 might also be a shared QTL: (1) analysis of linked cross-specific QTLs displayed borderline significance; (2) there is a different pattern of effect in different strain combinations that could be explained by background gene effect (ACI recessive, LEW dominant, and a tendency for DA/BN heterosis), and coding for combined-cross analysis could not distinguish different heterozygotes.
(coding according to phenotypic effect displayed no support for cross-specific QTLs); and (3) some evidence of linkage was detected in the vicinity of 50 Mb but not 40 Mb in the 7th and 10th generations of an intercross between DA/PVG, suggesting that these two strains might share the same variant at 40 Mb, whereas a QTL segregates between DA/PVG at 50 Mb, further supporting the possibility of two shared QTLs (P. Stridh-Igo, T. Olsson, K. Becanovic, unpublished data). The possibility of a third allelic variant, however, could not be excluded. This emphasizes a number of factors that should be taken into account when determining reproducible linkages.

Haplotype mapping has proven to be a powerful approach to map QTLs at an extremely high resolution both in mice [39] and in rats [40]. A combined-cross analysis can determine the allelic states on the basis of QTL effect in the individual crosses. More importantly, it can resolve multiple linked QTLs. These QTLs appear as a shared QTL, whereas they are distinct closely situated cross-specific QTLs. The outcome of analysis might be ambiguous in the case of QTLs that display very weak effects or QTLs that display distinct allelic effect in different crosses. However, parameters such as a significant difference in the lod score between the model assuming existence of linked QTLs versus the model assuming existence of a single QTL, accompanied by a reduction in the confidence interval, might assist in the resolution of linked QTLs. Consequently, the correct allelic state could be determined and the pattern of haplotype blocks in parental strains could be combined with QTL mapping data to refine the region. This information can significantly facilitate subsequent cloning of a susceptibility gene and help in delineation of the key polymorphism [41].

In conclusion, this is the first combined-cross analysis on data from F2 crosses in rat EAE. Our data demonstrate that a large fraction of EAE QTLs appear to be shared among different strain combinations and therefore reproduced by the combined-cross analysis, which supports their further investigation. There was a lack of power in previously performed F2 crosses, and larger populations in future studies together with combined-cross analysis are recommended. Moreover, application of combined-cross analysis provides instant benefits by means of improved power, resolution, and information on the allelic state of QTLs that can considerably facilitate gene identification.

Materials and methods

Theoretical background and practical steps in performing combined-cross analysis on several independent inbred line crosses have been described in detail by Li and colleagues [36]. In this study, data from three F2 crosses, summarized in Table 1, were combined and analyzed together. The genetic map and positions of all markers used in the three crosses were implemented from the rat genome sequence (http://www.ensembl.org/, version 29). The analyzed EAE phenotype was affection status of each rat, which represents the susceptibility to developing disease (defined as 1 for rats that developed EAE or 0 for rats that remained healthy). The final outcome of this phenotype is in fact binary; however, there is an underlying liability, a developing pathogenic response, which behaves as a real quantitative trait and once it reaches a threshold the disease ensues. The coherent correlation of affection status and different severity phenotypes, as well as their linkage to the same genome regions, demonstrated that these phenotypes are strongly dependent on each other [42,43]. We have, therefore, chosen affection status as a robust, representative disease phenotype because it can be accurately and objectively determined in different crosses. Alleles were recoded based on the parental phenotypes as follows: DA and LEW, which are susceptible strains, were coded as 1, whereas ACI, BN, and PVG, which are resistant strains, were coded as 3, and all heterozygotes were coded as 2. Genome-wide lod scores were generated using both the multiple imputation method with 64 simulations and the binary model [42,43]. Only data from the multiple imputation method are shown. The method is particularly suitable for this type of analysis since it simulates multiple versions of complete genotype information genome-wide using information in the marker genome while maintaining a considerable speed of computation [43]. The binary model, applied because of the binary nature of the analyzed EAE phenotype, generated comparable results (data not shown). For each F2 cross combination, an indicator variable (cross) was generated and included in the analysis as a covariate. To detect a shared QTL, i.e., a QTL that occurs in all of the crosses, the cross variable was used as an additive covariate. To detect a cross-specific QTL, i.e., a QTL that occurs in a subset of crosses, the cross variable was used as an interactive covariate to account for a QTL-by-cross interaction. A difference in the lod score between the analysis for a shared (LODadd) and that for a cross-specific QTL (LODint) indicates that the QTL is cross-specific. The p value for the statistically significant difference is computed from $\chi^2 = 2n(\text{LOD}_{\text{add}} - \text{LOD}_{\text{int}})$ with 4 degrees of freedom and corresponds to LOD$_{\text{add}}$-LOD$_{\text{int}}$>2.1 [36]. To detect linked QTLs, i.e., closely situated QTLs, a pair-scan analysis was performed with the cross as an interactive covariate. The difference between the lod scores generated from the cross-specific QTL analysis (LOD$_{\text{int}}$) and the pair-scan analysis bigger than 4.5 indicates linked QTLs in the region [36]. Significance levels, generated by 1000 permutations, were defined as 0.05 and 0.63 for significant and suggestive linkage, respectively [36]. A confidence interval of 95% for linkage was defined by bootstrapping (n=1000). All calculations were performed by GNU R 2.0.1 with the R/qtl package version 0.98-55 [44].

From the combined-cross data set of 658 F2 rats, five data sets of 200, 400, 600, 800, and 1000 rats were randomly generated in 10 replicates using a computer-assisted selection. Genome-wide lod scores were generated using the multiple imputation method with 64 simulations and the F2 cross as an additive covariate for each replicate, and significance levels were generated using 500 permutations for each data set. Average lod scores and standard deviations were derived for each data set.

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