Two Major Loci Arthritis Reveals an Interactive Effect by Gene-Mediated Effects on Collagen-Induced Arthritis

Anna-Karin B. Lindqvist, Martina Johannesson, Åsa C. M. Johansson, Kutty S. Nandakumar, Anna M. Blom and Rikard Holmdahl

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Backcross and Partial Advanced Intercross Analysis of Nonobese Diabetic Gene-Mediated Effects on Collagen-Induced Arthritis Reveals an Interactive Effect by Two Major Loci

Anna-Karin B. Lindqvist,2* Martina Johannesson,* Åsa C. M. Johansson,* Kutty S. Nandakumar,* Anna M. Blom,† and Rikard Holmdahl3*†

Genetic segregation analysis between NOD and C57BL strains have been used to identify loci associated with autoimmune disease. Only two loci (Cia2 and Cia9) had earlier been found to control development of arthritis, whereas none of the previously identified diabetes loci was of significance for arthritis. We have now made a high-powered analysis of a backcross of NOD genes on to the B10.Q strain for association with collagen-induced arthritis. We could confirm relevance of both Cia2 and Cia9 as well as the interaction between them, but we did not identify any other significant arthritis loci. Immune cellular subtyping revealed that Cia2 was also associated with the number of blood macrophages. Congenic strains of the Cia2 and Cia9 loci on the B10.Q background were made and used to establish a partial advanced intercross (PAI). Testing the PAI mice for development of collagen-induced arthritis confirmed the loci and the interactions and also indicated that at least two genes contribute to the Cia9 locus. Furthermore, it clearly showed that Cia2 is dominant protective but that the protection is not complete. Because these results may indicate that the Cia2 effect on arthritis is not only due to the deficiency of the complement C5, we analyzed complement functions in the Cia2 congenics as well as the PAI mice. These data show that not only arthritis but also C5-dependent complement activity is dominantly suppressed, confirming that C5 is one of the major genes explaining the Cia2 effect. The Journal of Immunology, 2006, 177: 3952–3959.

Identification of the molecular and cellular basis of the genes predisposing to development of human diseases will allow understanding of the actual biochemical and physiological pathways underlying the pathology. However, the uncovering of the genetic basis of most common human diseases has turned out to be involved with great challenges. Genotypic variation, creating phenotypic variation, as a result of evolutionary natural selection has been essential for all organisms to adapt to environmental conditions. Genotypic, and thereby phenotypic variation also determines individual susceptibility to common diseases. Therefore, virtually all common diseases have a complex etiology characterized by genetic as well as phenotypic heterogeneity, gene-gene and gene-environment interactions. The fast pace in which new genomic resources and technologies have been established has improved the chances of understanding the complexity and finding tools for systematic identification of genes underlying complex diseases (1).

Rheumatoid arthritis (RA)4 is one such common and complex disease. RA is a chronic inflammatory disease affecting ~1% of the human population. RA has a clear genetic predisposition with heritability estimated to ~60% (2). The genetic influence is to a significant degree associated with genes in the MHC. Efforts have been made to identify non-MHC susceptibility genes in human RA (exemplified by Refs. 3, 4).

A well-established strategy to explore the genetic causative factors and molecular mechanism for common disease is to use inbred animal models. There are a number of different animal models for RA (reviewed in Ref. 5). The most widely used model is collagen-induced arthritis (CIA) (reviewed in Ref. 6), which meets most of the criteria for diagnosis of RA. Both humoral and cellular immune mechanisms are involved in the induction and mediation of CIA. Various genetic linkage studies have until today located >30 genomic loci potentially harboring genes predisposing to CIA and other models for human RA in mice and rats. Subsequent isolation of the loci in congenic strains has provided fundamentally new knowledge on the genetic control of these diseases. The strategy has successfully identified two important genes regulating development of arthritis in mice, the MHC class II gene Aq controlling immune response (7) and the Ncf1 gene controlling oxidative burst (8, 9). Both of these genes are associated with T cell activation and arthritis severity. However, in many cases, the identification of the actual disease causing or modifying gene within the susceptibility loci has turned out to be more difficult than expected. The phenotype determined by a susceptibility locus often depends on the genetic background in which the locus is introduced, suggesting that genetic modifiers interact with the

*Medical Inflammation Research, Lund University, Lund, Sweden; and †Department of Laboratory Medicine, Section of Clinical Chemistry, Lund University, Malmö University Hospital, Malmö, Sweden

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2 Current address: Cartela AB, Box 709, Lund 22007, Sweden.

3 Address correspondence and reprint requests to Dr. Rikard Holmdahl, Medical Inflammation Research, BMC, 111, Lund University, Lund 22184, Sweden. E-mail address: rikard.holmdahl@med.lu.se

4 Abbreviations used in this paper: RA, rheumatoid arthritis; CIA, collagen-induced arthritis; PAI, partial advanced intercross; MAC, membrane attack complex; CII, collagen type II; LOD, logarithm of the odds; jnt, joint.
locus and thereby have a role in determining the functional expression of genetic variation (10).

Using animal models, the new strategy of partial advanced intercross (PAI) have recently been developed to investigate genetic interactions by repetitively crossing two congenic strains, both made on the same genetic background (11, 12). This strategy was used in the present study to investigate the interaction between the two arthritis susceptibility loci Cia2 and Cia9 in mice. We have previously identified the Cia2 and Cia9 loci as the main genetic factors influencing the arthritis development in a F2 intercross between the arthritis susceptible strain C57BL/10.Q (B10.Q) and the resistant NOD.Q strain (13). The Cia2 locus associated with CIA was also identified in (DBA/1 × SWR/J) F2 crosses (14, 15) and associated with arthritis in the KXB/N arthritis model in a cross between B6 and NOD (16). The Cia2 locus harbors a strong candidate gene for arthritis susceptibility, the complement component C5-encoding gene Hc. Both the NOD and the SWR/J strains carry a 2-bp deletion in an exon near the 5’ end of the Hc gene resulting in frame-shift and termination resulting in C5 protein deficiency (17, 18). The complement C5 plays multiple roles in the process of inflammation. Upon activation of C5, C5a fragment is released and acts as chemoattractant for neutrophils, monocytes, macrophages, and eosinophils, induces production of cytokines and other proinflammatory mediators, and enhances vascular permeability (19, 20). The other product of C5 activation, C5b, initiates assembly of membrane attack complex (MAC), which can further contribute to inflammation due to its ability to lyse cells and to induce drastic changes in cells when present at sublytic concentrations. The involvement of C5 in the pathogenic mechanisms of arthritis has been demonstrated in several reports (21–24). It has even been suggested that C5 sufficiency is an absolute requirement for CIA to develop (14, 22). The hypothesis that the Cia2 locus was almost exclusively mediated by a deficient C5 gene was, however, not compatible with some of the reported findings. First, mice with NOD-derived Cia2 could develop arthritis, although at low incidence rate, and second, mice with Cia2 heterozygosity, with undisturbed C5 levels in the serum, had a dramatically reduced arthritis severity (13). In addition, the Cia2 loci seemed to influence the functional expression of the Cia9 locus on chromosome 1, indicating an interactive influence. To resolve these questions, we made an extensive backcross experiment to investigate the possibility that there are other dominant loci that could interact. Furthermore, we established congenic strains of the Cia2 and Cia9 loci and made a PAI between them to pinpoint the interactive effects. Finally, we analyzed the role of Cia2 in modulating complement functions to provide an explanation for a possible C5-mediated effect.

Materials and Methods

Mice

Mice were bred and kept in the animal house of Section for Medical Inflammation Research, Lund University. The mice were kept in a climate-controlled environment with 12-h light/dark cycles and fed with standard rodent chow and water ad libitum (as defined at www.inflam.lu.se). The congenic strain for the Cia2, B10.Q(NOD-Cia2), was constructed by the speed congenic technique (25) as described previously (13). For this study, the congenic strain had been backcrossed to B10.Q strain for additional 5 generations, yielding in total 10 generations of backcrossing. The congenic fragment from NOD.Q was ~54-Mb long, ranging from D2Mit116 to D2Mit91. The congenic strain for the Cia9 locus (B10.Q(NOD-Cia9)) was constructed using the same technique as the Cia2 congenic (25). The congenic fragment from NOD.Q was ~150-Mb long, ranging from D1Mit25 to D1Mit17 (see Fig. 6).

In a PAI set-up, in similarity with Ref. 11, Cia2 and Cia9 congenic mice were intercrossed. F2 mice were genotyped, and selected mice were used for breeding. For each following generation, mice were selected for next generation of breeding, and mice not used for breeding were subjected to CIA induction. The aim was to produce all possible combinations of genotypes at the location of the linkage peaks within the Cia2 and Cia9 loci, respectively. Data were collected from 212 PAI mice, 96 Cia2 single and subcongenics, 62 Cia9 single and subcongenics, and 111 wild-type B10.Q mice in five CIA experiments. Single congenics and wild-type mice were used to control for disease variation in the separate CIA experiments. All experiments involving animals were approved by the local ethical committee.

Genotyping

Microsatellite markers were purchased from Interactiva Biotechnology. The order of the markers was based on the map available from the Jackson laboratory (www.jax.org).

Backcross.

A full genomic scan was performed on 395 backcross animals using 162 microsatellite markers. The complete list of markers is available on (www.inflam.lu.se).

All markers were assayed by PCR as follows: Genomic DNA (15 ng) was amplified in a final volume of 9 μl containing (200 μM), MgCl2, (1.5 mM), primer (1.5 pmol of each), and TaqGold DNA polymerase (0.2 U) (PerkinElmer Biosystems). The forward primer was labeled with a fluorescent dye. Amplification conditions were as follows: 95°C for 1.1 min, followed by 33 cycles of 95°C for 30 s, 55°C for 75 s, 72°C for 75 s, and a final extension at 72°C for 7 min. The reactions were performed using an ABI (Applied Biosystems) 377 sequencer (Applied Biosystems), followed by automated pooling of the products. PCR products were size-fractionated on 4% polyacrylamide gels, on an ABI 377 sequencer, and the size of the fragments were determined using the Genescan software version 3.1 (Applied Biosystems) with TAMRA GS-350 or 500 as internal size standard.

Partial advanced intercross.

DNA was prepared from tail biopsies by a quick alkaline lysis protocol (26). Briefly, tail biopsies were incubated in 50 mM NaOH for 2 h at 95°C, vortexed, and neutralized in 1 M Tris-HCl (pH 8.0). After centrifugation, the supernatant was used for PCR. Genotypes from 502 PAI mice were analyzed from 11 markers spanning 46.0–189.3 Mb on chromosome 1 and 6 markers from 12.0 to 38.2 Mb on chromosome 2 (see www.inflam.lu.se). Genotypes from 162 microsatellite markers were analyzed.

PCR was performed with 10 ng of DNA in a reaction volume of 10 μl containing the following: 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2, 0.3 μM forward and reverse primer (MWG-Biotec and Applied Biosystems), 100 μM dNTP (Amersham Biosciences), and 0.25 U Taq (Amersham Biosciences). Forward primers were labeled with fluorescent dyes. PCR was performed in a thermal cycler (MJ Research) under the following amplification conditions: denaturation at 94°C for 2.5 min, annealing at 56°C for 45 s, polymerization at 72°C for 1 min, followed by 30 cycles of 94°C for 30 s, 56°C for 45 s, and 72°C for 1 min. The final cycle ended by elongation at 72°C for 6 min. PCR products were analyzed on a MegaBACE 1000 (Amersham Biosciences), according to the manufacturer’s protocol.

Induction and evaluation of CIA

At the age of 10–12 wk, mice were immunized at the base of the tail with 100 μg of rat collagen type II (CII) emulsified in CFA (Difco) in a volume of 100 μl. At 35 days postimmunization, the mice were given a boost injection of 50 μg of CII emulsified in IFA (Difco) in 100 μl. Arthritis was evaluated blindly using a scoring system, as described previously (27), based on the number of inflamed joints in each paw. Briefly, each toe and knuckle was given a score of 1 (total 10/limb), and inflamed wrist of ankle was given a score of 5, giving a maximum score per limb of 15 and a maximal score per mouse of 60. Severity of arthritis was evaluated using a maximum disease score and as accumulated score (area under the curve), generated by calculating the sum of the disease scores for each mouse, respectively.

Measurement of anti-CII IgG levels

Mice were bled at day 35 after induction of CIA, and the level of mouse anti-CII IgG Abs (μg/ml) were measured in the sera, using ELISA as previously described (28).

Detection of complement factor 5 in sera

The levels of complement factor 5 in sera were detected using the ELISA as described previously (29). Sera (from day 35 after induction) were added to 96-well plates (Costar, Corning) coated with a chicken anti-mouse C5 Ab. To detect C5 levels, a biotinylated chicken anti-mouse Ab was used followed by a streptavidin alkaline phosphatase (Jackson ImmunoResearch).
Laboratories) incubation. Parainitrophenol was used as a chromogenic substrate, and the absorbance was determined in a Titertek multiscan spectrophotometer.

Abs for flow cytometry

For staining of surface epitopes, the following mAbs were used: anti-CD4 (H129.19) and anti-CD8 (53.6.7), purchased from BD Pharmingen. Anti-B220 (RA3-6B2), anti-CD4 (CT-CD4), and anti-CD8 (CT-CD8) (all three from Caltag Laboratories), anti-CD11B (M1/70), anti-FcγRII/III (2.4.G2.), and anti-CD3ε (145-2C11) were purified from culture supernatants by affinity chromatography on protein G-Sepharose and conjugated.

Tissue preparation and in vitro flow cytometry

At the end of the experiment spleens were removed, and cell suspension was prepared in PBS by mechanically mashing the tissue. Cells were washed and resuspended in staining buffer containing 0.5% BSA (Sigma-Aldrich) and 0.01% NaN₃ in PBS. A total of 1–2 × 10⁶ cells was stained for various surface epitopes for 20 min at 4°C. An anti-Fc γR used to inhibit Ab binding to the Fc γR. A typical forward and side scatter gate for activated lymphocytes was set to exclude dead cells and aggregates. Typically, 10⁵ events from the lymphocyte gate were collected and analyzed using a FACS Sort (BD Biosciences) and BD Biosciences software. Quadrant and histogram statistics were placed on the basis of the staining of the negative controls. Less than 0.5% positively stained cells was not regarded as significant.

Complement assay

Activity of the alternative pathway was studied using hemolytic assay for measurement of the alternative pathway including activity of C5. Rabbit erythrocytes were washed twice in Mg₂EGTA buffer (2.5 mM veronal buffer (pH 7.3), 70 mM NaCl, 140 mM glucose, 0.1% gelatin, 7 mM MgCl₂, and 10 mM EGTA) and resuspended at 0.5 × 10⁶ cells/ml. Mouse serum (5 μl) was incubated with 25 μl of erythrocyte suspension in total volume of 100 μl of Mg₂EGTA buffer. After 4 h of incubation at 37°C, the degree of lysis was determined by spectrophotometric measurement of the amount of released hemoglobin at 405 nm. For each sample, an absorbance provided by serum itself was subtracted, and the values are shown as Ze, which equals the natural negative logarithm of 1 – percentage of lysis.

Statistical and linkage analysis

Linkage analysis was performed using R/qtl QTL mapping software (30) in R (R Development Core Team, 2004). The genome scan was performed using scanone (imputation model) (31) under the assumption that sex influences the arthritis development as an interactive covariate. The chromosome X was omitted. Arthritis severity was analyzed as maximum arthritis score and accumulated arthritis score (area under the curve). QTL analysis was calculated under the assumption that gender influenced the arthritis development as an interactive covariate. To investigate gene interactions, two-dimensional genome scan with a two-QTL model was performed. In the analysis, LOD scores are calculated testing the full model, in the presence of covariates, to each of two alternatives: The joint LOD (LODjoint) score compares the full model to the null model, and the epistasis LOD score (LODjoint) compares the full model to the additive model. The full model is as follows: y = m + [q₁ + [q₂ + [q₁ × q₂] + Ag + Zd[q₁] + Zd[q₂] + Zd[q₁ × q₂] + e], where q₁ and q₂ are the unknown genotypes at two locations, A is a matrix of covariates, and Z is a matrix of QTL-interacting covariates. The null model is as y = m + Ag + e + the additional model is as y = m + [q₁ + [q₂ + [Ag + Zd[q₁] + Zd[q₂] + e]. Empirical significance levels (70, 95, and 99%) were established by permutation tests (1000 permutations) (32) for each phenotype.

The marker map covered the NOD.Q × B10.Q backcross genome at an 20 cM intermarker distance.

Other statistical analyses were performed using Mann-Whitney U test for comparisons between groups. Differences were regarded as significant if the p value was <0.05.

Results

The Cia9 and Cia2 loci are the main loci influencing arthritis development in crosses between the B10.Q and NOD.Q

We have previously identified Cia9 and Cia2 as loci regulating arthritis severity in a F₂ intercross between the B10.Q and NOD.Q strains (13). Because we were surprised that we did not find any other loci, in particular loci earlier identified to be associated with diabetes using crosses with the same genetic backgrounds, we designed a powerful backcross experiment. We produced 395 (B10.Q × NOD.Q) × B10.Q backcross mice in an effort to find additional modifying loci and to define the relative importance of Cia2 and Cia9. As expected, the arthritis developed variably from none to a severe acute disease that in most affected animals resulted in joint deformation. Genotypes from 162 markers genomewide were analyzed, and a genetic linkage analysis was performed. Again, we found that the Cia2 and Cia9 were the strongest loci affecting the arthritis development, as in the earlier F₂ intercross (Table I). To our surprise, only one additional locus was identified, a locus in the centromeric part of chromosome 10 (7.0 cM), that influenced arthritis severity (LOD = 2.94) (Table I). No other loci were detected in the present backcross. We conclude that in crosses between the NOD and C57BL/10 genomes, the polygenic segregation is mainly dependent on only two dominant loci, the Cia9 locus promoting arthritis and Cia2 protecting when originating from the NOD strain.

Immune response and cell subphenotyping

We could not identify any loci linked to immune response against CII measured as levels of anti-CII Abs in the present backcross (data not shown). Neither did we detect any difference in anti-CII Ab production between B10.Q-NOD-Cia2 congenic mice and wild-type B10.Q (data not shown). These data confirmed our previous finding of no loci linkage to anti-collagen Ab production in

Table I. Regions of linkage to arthritis incidence and severity identified in (B10.Q × NOD.Q) × B10.Q backcross

<table>
<thead>
<tr>
<th>Arthritis Phenotypes</th>
<th>Locus</th>
<th>Marker</th>
<th>Position (cM)</th>
<th>Position (Mb)</th>
<th>LOD Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incidence</td>
<td>Cia9</td>
<td>D1mit349</td>
<td>81.6</td>
<td>158.5</td>
<td>2.77*</td>
</tr>
<tr>
<td></td>
<td>Cia2</td>
<td>D2mit296-D2Mit91</td>
<td>18.0–37.0</td>
<td>31.1–66.7</td>
<td>4.25***</td>
</tr>
<tr>
<td>Maximum score</td>
<td>Cia9</td>
<td>D1mit349</td>
<td>81.6</td>
<td>158.5</td>
<td>6.35***</td>
</tr>
<tr>
<td></td>
<td>Cia2</td>
<td>D2mit296</td>
<td>18.0</td>
<td>31.3</td>
<td>8.62***</td>
</tr>
<tr>
<td>Accumulated arthritis score</td>
<td>Cia9</td>
<td>D1mit349</td>
<td>81.6</td>
<td>158.5</td>
<td>5.90***</td>
</tr>
<tr>
<td></td>
<td>Cia2</td>
<td>D2mit296-D2Mit91</td>
<td>18.0–37.0</td>
<td>31.1–66.7</td>
<td>5.82***</td>
</tr>
</tbody>
</table>

*a* QTL analysis was performed using R/qtl (30). Significance levels were established by permutation. Arthritis severity was expressed as maximal arthritis score and as accumulated arthritis scores along the time course of the experiment.

*b* Genetic position according to Mouse Genome Informatics (http://www.informatics.jax.org/).

*c* Physical position according to Ensembl Mouse Genome Server (http://www.ensembl.org/Mus_musculus/).

*d* LOD score calculated allowing gender as an interactive covariate using R/qtl (http://www.biostat.jhsph.edu/~kbroman/qtl/). Significance levels: *, p (0.90); ***, p (0.99).
the F₂ intercross between B10.Q and NOD.Q (13). Instead, to further dissect the mechanism of arthritis in the backcross, we characterized the mice regarding distribution of immune and inflammatory cells in blood. Frequency distribution was determined by flow cytometry on spleen cells taken at the end point of the experiment. The frequency of the different cell types were used as quantitative traits in linkage analysis to identify loci determining the cell distributions. We identified a locus on chromosome 12 involved in the distribution of both T and B cells (Table II). The NOD allele at D12Mit76 reduced the frequency of B cells and thereby the ratio of B cells:T cells (data not shown). The chromosome 12 locus also influenced the ratio of CD4-positive to CD8-positive T cells by the NOD allele increasing the ratio. The Cia2 locus influenced the frequency of CD11b-positive cells (Table II), and the higher expression of CD11b-positive cells correlated with arthritis development \( (\rho = 0.011) \) (Fig. 1). Moreover, the arthritis-suggestive locus on chromosome 10 was associated with a decreased frequency of CD11b-positive cells in blood. However, the Cia9 locus did not influence the frequency or distribution of any of the investigated cells.

**Arthritis-promoting Cia9 locus partly overcomes the inhibitory effect of Cia2**

In the backcross, the linkage to Cia2 was significantly associated with reduction of arthritis development. If the mice were homozygous B10.Q at the Cia9 locus, the effect of B10.Q/NOD.Q heterozygosity at Cia2, mediated almost complete inhibition of arthritis development (thick line, Fig. 2). However, introduction of one NOD allele at Cia9 partially overcame the inhibitory effect of Cia2, enabling the development of arthritis (thin line, Fig. 2). The interaction between the two loci was highly significant (LOD score of 16.1 (LODjnt)).

*The interaction between Cia9 and Cia2 in arthritis development confirmed in a PAI*

To confirm the linkage to Cia2 and Cia9, the fragments were introgressed on B10.Q, and subsequently the congenic strains were used to establish a PAI cohort following an earlier outlined strategy (11) (Fig. 3). Using this strategy, we obtained mice with combinations of the genotypes of the two loci to determine differences in arthritis incidence and severity, without influences from the rest of the genome. The dominant protective effect of Cia2 was clearly confirmed in the PAI, seen by almost complete inhibition of arthritis in mice carrying one or two NOD.Q alleles at the Cia2 locus (Table III and Fig. 4B, △ and △). However, in the mice carrying one or two NOD alleles at Cia9 in combination with the Cia2 alleles, the complete inhibition mediated by Cia2 was broken. Even mice homozygous for NOD at Cia2 could develop disease if combined with the disease-promoting allele at Cia9 (Fig. 4B, □). The Cia9 by itself was influencing the disease by increasing the arthritis severity in the mice homozygous and heterozygous for NOD alleles at the peak marker (D1Mit270) of Cia9, located close to the FcR region (Fig. 4B, ○ and ■) as compared with B10.Q wild-type mice (Fig. 4B, ▲). Interestingly, the inheritance was different at the more centromeric part of the congenic region on chromosome 1 (at marker D1Mit14) where heterozygosity enhanced the disease, whereas homozygosity of the NOD allele in fact protects against arthritis (Fig. 4A). This argues for the presence of at least two genes in the Cia9 fragment.

**The Cia2 locus dominantly suppresses complement function and arthritis development in the B10.Q.NOD-Cia2 congenic strain**

Using the B10.Q.NOD-Cia2 congenic mice, we could confirm the observation from the backcross that the Cia2 locus completely

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**Table II. Regions of linkage to cell distribution in (B10.Q × NOD.Q) × B10.Q backcross**

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>Locus</th>
<th>Marker</th>
<th>Position (cM)</th>
<th>Position (Mb)</th>
<th>LOD Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cell:T cell ratio (B220/CD3)</td>
<td></td>
<td>D12Mit76</td>
<td>43.0</td>
<td>82.6</td>
<td>2.88*</td>
</tr>
<tr>
<td>B cells (B220)</td>
<td></td>
<td>D12Mit76</td>
<td>43.0</td>
<td>82.6</td>
<td>4.41***</td>
</tr>
<tr>
<td>T cells (CD3)</td>
<td></td>
<td>D1Mit89</td>
<td>63.1</td>
<td>124.4</td>
<td>3.08*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D13Mit78</td>
<td>75.0</td>
<td>115.8</td>
<td>3.15*</td>
</tr>
<tr>
<td>CD4:CD8 ratio</td>
<td></td>
<td>D8mit180</td>
<td>34.0</td>
<td>74.0</td>
<td>4.60***</td>
</tr>
<tr>
<td>CD8</td>
<td></td>
<td>D12Mit76</td>
<td>43.0</td>
<td>82.6</td>
<td>4.96***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D14Mit11</td>
<td>0.7</td>
<td>8.7</td>
<td>3.16**</td>
</tr>
<tr>
<td>CD11b</td>
<td>Cia2</td>
<td>D2mit98</td>
<td>50.3</td>
<td>98.6</td>
<td>2.69*</td>
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<tr>
<td></td>
<td></td>
<td>D2mit296</td>
<td>18.0</td>
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<td>3.25**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D4Mit4</td>
<td>12.1</td>
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<td></td>
<td></td>
<td>D10Mit86</td>
<td>17.0</td>
<td>24.3</td>
<td>5.11***</td>
</tr>
</tbody>
</table>

*QTL analysis was performed using R/qtl (30). Significance levels were established by permutation. Frequency distribution of cells in serum was determined by flow cytometry. Arthritis severity was expressed as maximal arthritic score and as accumulated arthritic scores along the course of the experiment.

Genetic position according to Mouse Genome Informatics (http://www.informatics.jax.org/).

Physical position according to Ensembl Mouse Genome Server (http://www.ensembl.org/Mus_musculus/).

LOD score calculated allowing gender as interactive covariate using R/qtl (http://www.biostat.jhsph.edu/~kbroman/qtl/).

Significance levels: *, \( p (0.90); **, p (0.95); *** , p (0.99). \)
suppresses arthritis development in a dominant fashion (Fig. 5), because none of the homozygous or heterozygous Cia2 congenic mice developed CIA. Within this fragment the NOD allele of C5 (Hc) has a mutation prohibiting C5 expression, and because C5 has been suggested to be of crucial importance for arthritis susceptibility, it is the major candidate gene. The levels of C5 in blood were reduced in mice heterozygous for the NOD allele of C5, although not statistically significantly lower than in wild-type B10.Q mice (C5 sufficient) (Fig. 6). We then investigated effect of Cia2 on functional effects of complement. We analyzed the complement activity using functional hemolytic assay, dependent on the presence of C5, after arthritis induction but before disease onset as well as during active inflammation (days 0, 35, and 70). As expected, we could demonstrate no complement C5-dependent lysis in the homozygous Cia2 congenics (Fig. 7). Interestingly, at the time of induction (day 0) as well as day 35 after disease induction, the effect on C5-dependent lysis was significantly less pronounced in the heterozygous Cia2 congenics compared with

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Incidence (%) (^a)</th>
<th>Maximum Arthritic Score (^c)</th>
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<tr>
<td>a</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>b</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>h</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>h</td>
<td>2</td>
<td>16</td>
</tr>
</tbody>
</table>

\(^{a}\) Incidence of arthritis at 35, 49, 58, and 70 days, respectively, after disease induction. The backcross mice are grouped based on their genotype of Cia9, given by the marker D1Mit270, in combination with the genotype at Cia2, given by the marker in the Hc gene.

\(^{b}\) Genotypes given as a, NOD.Q homozygous; b, B10.Q homozygous; h, heterozygous.

\(^{c}\) Incidence of arthritis at 35, 49, 58, and 70 days, respectively, after immunization of CII.

\(^{d}\) Maximum arthritic score from day of immunization until end of experiment at day 70.
The wild-type B10.Q (Fig. 7). This finding supports a dominant protective effect of the mutated C5 gene because this led to a decreased functional complement activity. However, it does not exclude additional modifying effects by other polymorphic genes within the Cia2 fragment.

Discussion

CIA is a polygenic disease in mice, but in this study we show that only two loci, located on chromosome 2 (Cia2) and chromosome 1 (Cia9), controlled the development of arthritis in a highly powered backcross between NOD and C57BL strains. Together with earlier published intercross data, this excludes a major influence of all of the loci-determining diabetes that has been identified using F2 crosses between these backgrounds. However, this surprising finding might not exclude other major loci, because it is possible that such loci could be silenced by the interactive effect of genes in linkage, and that the level of recombination density is not high enough in a F2 intercross to liberate these influences. Such effects have indeed been shown to operate using PAI, where a higher density of recombination was studied on predetermined quantitative trait loci. However, it is also possible that the disease pathogenesis of diabetes and arthritis are dramatically different. Possibly, this could be due to the fact that the studied diabetes is a spontaneous disease, whereas the presently studied arthritis is induced by immunization. However, both loci identified seem to operate irrespective of the immunization because they affect the effector phase rather than the priming phase of arthritis. In fact, the Cia2 locus is dependent on the effect of the C5 gene, which is known to have no major influence in diabetes. It is more surprising that there are no similarities between diabetes and arthritis on the centromeric Idd5 locus on chromosome 1 involving CTLA4, where diabetes but not arthritis is associated (33), and the telomeric Cia9 locus involving the SLAM locus and the FcR complex, where both arthritis and lupus show strong linkage, whereas no association with diabetes is found (13, 34). However, both Cia2 and Cia9 loci are large and were in addition shown to interact with each other. To further confirm the genetic interaction and facilitate positional identification of the underlying genes, we made a PAI between the Cia2 and Cia9 congenic strains. We confirmed that...
the arthritis resistance allele from NOD.Q at Cia2 alone reduced incidence of arthritis, but if one arthritis-promoting allele from NOD.Q at Cia9 was introduced, the mice could still develop disease. Consequently, in these mice arthritis is developed despite the lack of functional C5. Although it has been suggested previously that C5 sufficiency is an absolute requirement for CIA to develop (14, 22), we showed that the arthritis-promoting effect of the Cia9 loci may decrease the threshold to the level where arthritis is developed due to a parallel, complement-independent pathway. This points toward multiple possible pathways by which the arthritis can develop, and that the combination of the genetic factors will determine which pathway is initiated.

Our results indicate that the Cia2 locus in the Cia2-congenic strain inhibits arthritis in a dominant fashion, and we show that the function of C5 is disturbed in the heterozygous animals. Although the levels of C5 in serum are not significantly reduced in heterozygous animals, we conclude that the levels of C5 in serum are not significantly reduced in heterozygous animals. Although these complement inhibitors to be used in the clinic. Although inhibitors of C3-convertase (enzymatic complex activating C3 into C3b and C3a) may protect against arthritis, they are only moderately efficient (21) or may cause more side effects than therapy aiming at inhibition of C5. For example, opsonization with C3b is the main mechanism by which complement contributes to phagocytosis of pathogenic microorganisms. The hypothesis that C5a and/or MAC are main contributors to the disease is further supported by beneficial effects of oral C5aR antagonist in treatment of arthritis in rat (38) as well as increased arthritis severity in mice lacking the MAC inhibitor, CD59 (39). Interestingly, in the PAI experiment, the level of complement activity was up-regulated at day 70, which is consistent with the fact that several complement factors are acute phase proteins up-regulated upon inflammation. Although our study shows the genetic complexity of development of arthritis, it seems clear that C5 deficiency is involved, explaining a large part of the arthritis variance and confirming C5 as an important target for anti-inflammatory therapy of arthritis.

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Disclosures
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References