The Journal of Immunology

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J Immunol 2006; 177:3952-3959; ;
doi: 10.4049/jimmunol.177.6.3952
http://www.jimmunol.org/content/177/6/3952

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Backcross and Partial Advanced Intercross Analysis of Nonobese Diabetic Gene-Mediated Effects on Collagen-Induced Arthritis Reveals an Interactive Effect by Two Major Loci[1]

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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 genome 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

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Received for publication January 6, 2006. Accepted for publication June 22, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the Swedish Research Council, Swedish Foundation for Strategic Research (INGVAR), Greta and Johan Kock's Research Foundation, King Gustav V's 80th Anniversary Foundation, Anna Greta Crafoord (Crafoord) Foundation, Kock Foundation, Österlund Foundation, Swedish Association against Rheumatism, Swedish Medical Research Council, Strategic Research Foundation, and the European Union projects MUGEN and AUTOCURE.

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[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.

0022-1767/06/$02.00

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 F_2 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) F_2 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 D1Mit235 to D1Mit17 (see Fig. 6).

In a PAI set-up, in similarity with Ref. 11, *Cia2* and *Cia9* congenic mice were intercrossed. F_2 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 dNTP (200 μM), $MgCl_2$ (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 877 Integrated Thermal cycler (Applied Biosystems), followed by an automatic pooling of the products. The 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 $MgCl_2$, 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 1:1 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 as 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. Paranitrophenol 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% NaN_3 in PBS. A total of 1–2 $\times$ 10^6 cells was stained for various surface epitopes for 20 min at 4°C. An anti-FcγRII/III Ab was used to inhibit Ab binding to the FcγR. A typical forward and side scatter gate for activated lymphocytes was set to exclude dead cells and aggregates. Typically, 10^4 events from the lymphocyte gate were collected and analyzed using a FACSort (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 Mg-EGTA buffer (2.5 mM veronal buffer (pH 7.3), 70 mM NaCl, 140 mM glucose, 0.1% gelatin, 7 mM $MgCl_2$, and 10 mM EGTA) and resuspended at 0.5 $\times$ 10^9 cells/ml. Mouse serum (5 μl) was incubated with 25 μl of erythrocyte suspension in total volume of 100 μl of Mg-EGTA 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 Z, 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 arthritic score and accumulted arthritic 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 (LODjnt (joint)) score compares the full model to the null model, and the epistasis LOD score (LODint) compares the full model to the additive model. The full model is as follows: ($y = m + b[q1] + b[q2] + b[q1 \times q2] + Ag + Zd[q1] + Zd[q2] + Zd[q1 \times q2] + e$), 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 $y = m + Ag + e$ and the additional model is as $y = m + b[q1] + b[q2] + Ag + Zd[q1] + Zd[q2] + e$. 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 $\times$ 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 F_2 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 $\times$ NOD.Q) $\times$ 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 genome-wide 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 F_2 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 $\times$ NOD.Q) $\times$ B10.Q backcross*[a]

Arthritis Phenotypes	Locus	Marker	Position (cM)[b]	Position (Mb)[c]	LOD Score[d]
Incidence	*Cia9*	D1mit349	81.6	158.5	2.77*
	Cia2	D2mit296-D2Mit91	18.0–37.0	31.1–66.7	4.25***
Maximum score	*Cia9*	D1mit349	81.6	158.5	6.35***
	Cia2	D2mit296	18.0	31.3	8.62***
		D10Mit50	7.0		2.94*
Accumulated arthritis score	*Cia9*	D1mit349	81.6	158.5	5.90***
	Cia2	D2mit296-D2Mit91	18.0–37.0	31.1–66.7	5.82***
		D10Mit50	7.0	17.5	2.54*

[a] QTL analysis was performed using R/qtl (30). Significance levels were established by permutation. Arthritis severity was expressed as maximal arthritic score and as accumulated arthritic 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 (0.95); ***, p (0.99).

the F_2 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 ($p = 0.011$) (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.

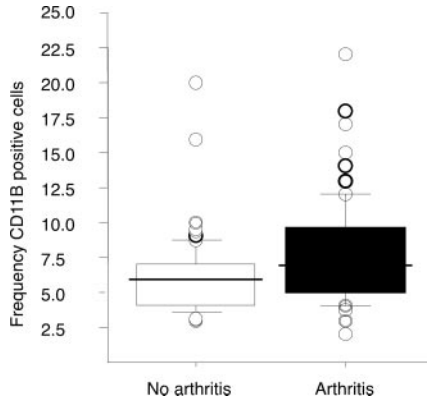

FIGURE 1. Higher expression of CD11b-positive cells in arthritic mice. The frequency of CD11b cells in spleen of arthritic backcross mice were higher than in nonarthritic mice ($p = 0.0011$). Total spleen cells were prepared at the end of the arthritis experiment.

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. 4*B*, △ 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. 4*B*, □). 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. 4*B*, ● and ■) as compared with B10.Q wild-type mice (Fig. 4*B*, ▲). 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. 4*A*). 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

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

Phenotypes	Locus	Marker	Position (cM)[b]	Position (Mb)[c]	LOD Score[d]
B cell:T cell ratio (B220/CD3)		D12Mit76	43.0	82.6	2.88*
B cells (B220)		D12Mit76	43.0	82.6	4.41***
T cells (CD3)		D1Mit89	63.1	124.4	3.08*
		D13Mit78	75.0	115.8	3.15*
CD4:CD8 ratio		D8mit180	34.0	74.0	4.60***
		D12Mit76	43.0	82.6	4.96***
		D14Mit11	0.7	8.7	3.16**
CD8		D2mit98	50.3	98.6	2.69*
CD11b	*Cia2*	D2mit296	18.0	31.3	3.25**
		D4Mit4	12.1	37.0	3.08*
		D7Mit246	15.0	22.0	3.43**
		D10Mit86	17.0	24.3	5.11***

[a] 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 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 interactive covariate using R/qtl (⟨http://www.biostat.jhsph.edu/≈kbroman/qtl/⟩). Significance levels: *, p (0.90); **, p (0.95); ***, p (0.99).

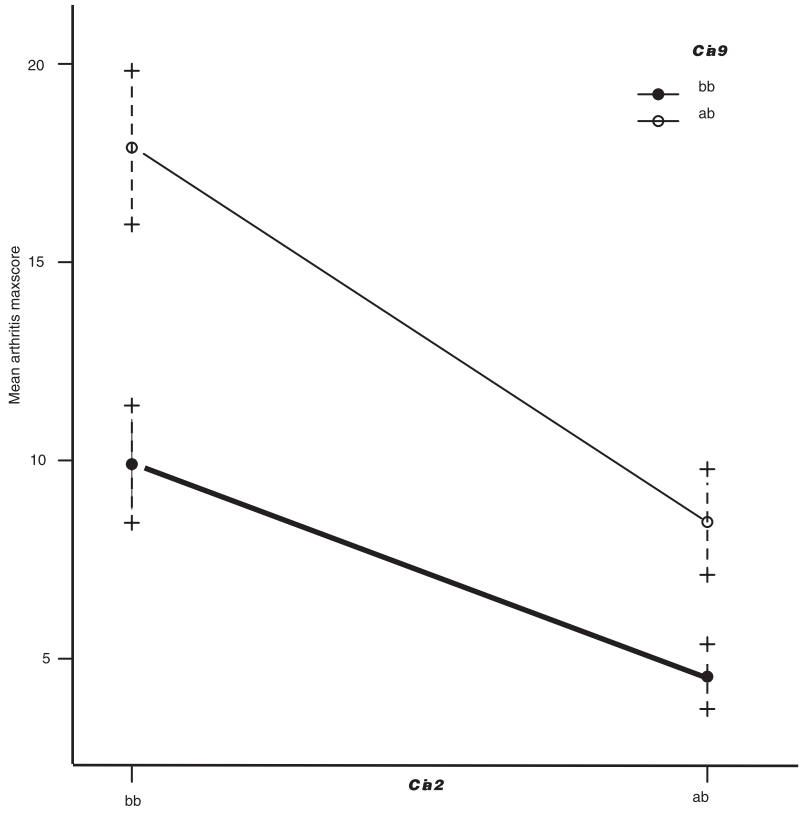

FIGURE 2. The arthritis-promoting *Cia9* locus partly overcomes the reducing effect of *Cia2* in the backcross. Interaction plot illustrating the influence of the *Cia9* locus over the *Cia2* locus in arthritis development in the backcross. In mice homozygous for the B10.Q allele at *Cia9* (here illustrated by the marker D1Mit349), *Cia2* (here D2Mit296) almost completely inhibits arthritis development (thick line). In mice B10.Q/NOD.Q heterozygous at *Cia9*, the arthritis-promoting effect of the *Cia9* locus from the NOD allele partly overcomes the inhibition by *Cia2* (thin line) with a highly significant LOD score of 16.1 (LODjnt = 16.1); bb, B10.Q homozygous genotype; ab, heterozygous.

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* congenic mice (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

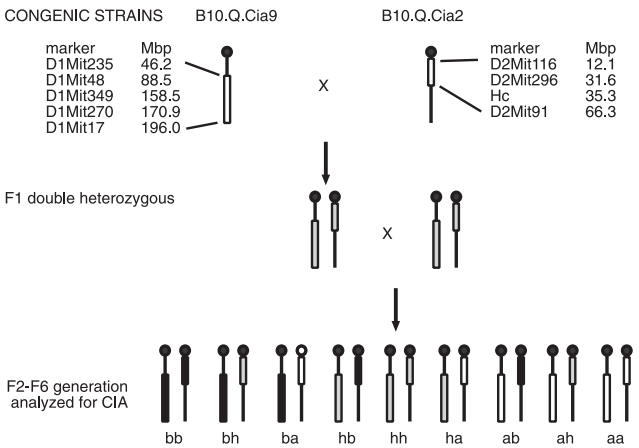

FIGURE 3. The PAI between *Cia2* and *Cia9* congenics. The *Cia9* congenic strain (B10.Q.NOD-*Cia9*) was produced from (B10.Q × NOD.Q) B10.Q offspring crossed to B10.Q and subsequently further backcrossed for nine generations. The *Cia2* congenic strain (B10.Q.NOD-*Cia2*) was produced (13) and further backcrossed additionally five generations to B10.Q. For the PAI, the B10.Q.NOD-*Cia9* and B10.Q.NOD-*Cia2* congenics were intercrossed to produce double heterozygous F_1 mice, which were intercrossed to produce F_2 animals that were selected for further intercross breeding based on genotypes of the *Cia9* and *Cia2* locus. The aim was to produce all combinations of genotypes at the *Cia2* and *Cia9* loci and gain recombinations to reduce the fragments of interest.

Table III. *Incidence of arthritis in the partial-advanced intercross*[a]

Genotype		Incidence (%)[c]				
Cia9 D1Mit270[b]	*Cia2* *Hc*[b]	Day 35	Day 49	Day 58	Day 70	Maximum Arthritic Score[c]
a	a	0	0	0	20	5
a	b	4	23	15	30	60
a	h	0	16	12	28	30
b	a	0	0	4	7	2
b	b	5	34	40	52	52
b	h	1	4	3	9	4
h	a	0	12	8	16	29
h	b	3	26	29	35	45
h	h	2	15	32	34	37

[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.

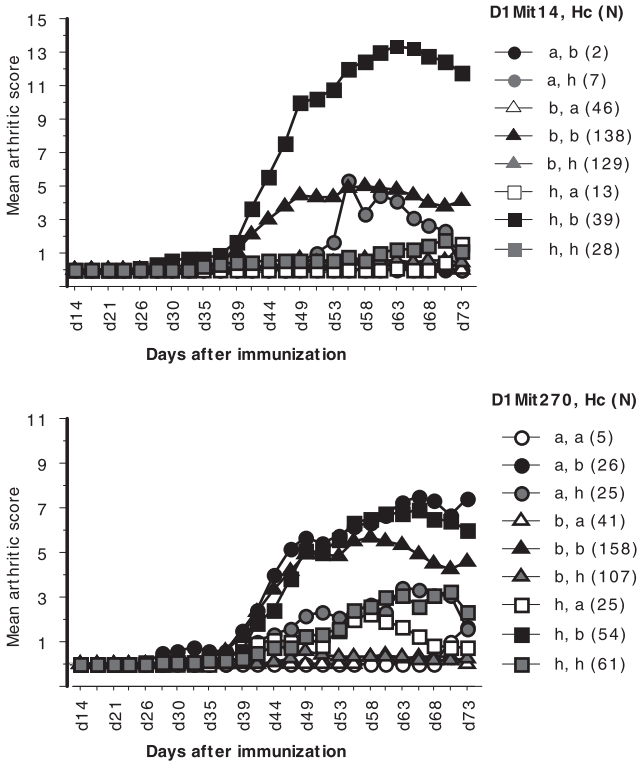

FIGURE 4. Arthritis severity in mice from the PAI. In the PAI, it was confirmed that the arthritis-promoting effect of *Cia9* overcomes the complement deficiency of the *Cia2* locus originating from NOD.Q, enabling mice heterozygous and homozygous NOD.Q in *Cia2* to develop arthritis. The disease curve is for the mice in the PAI, which were followed for 73 days after collagen II injection. The arthritis severity is given as mean arthritic scores (for all mice). Genotypes given as follows: a, NOD.Q homozygous; b, B10.Q homozygous; and h, heterozygous. The shapes of the symbols illustrate the *Cia9* genotype: circles, homozygous NOD; triangles, homozygous B10.Q; squares, heterozygous. The colors of the symbols illustrate the genotype at *Cia2*: empty symbols, homozygous NOD; black symbols, homozygous B10.Q; gray symbols, heterozygous. Number of mice per group is given within brackets. Data are based on the centromeric marker of *Cia9* (D1Mit14, at 157 Mbp from the centromer), or on a telomeric marker of *Cia9* (D1Mit270 at 172 Mbp from the centromer).

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 F_2 crosses between these backgrounds. However, this surprising finding might not exclude other major loci, because it is possible that such locus could be silenced by the interactive effect of genes in linkage, and that the level of recombination density is not high enough in a F_2 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

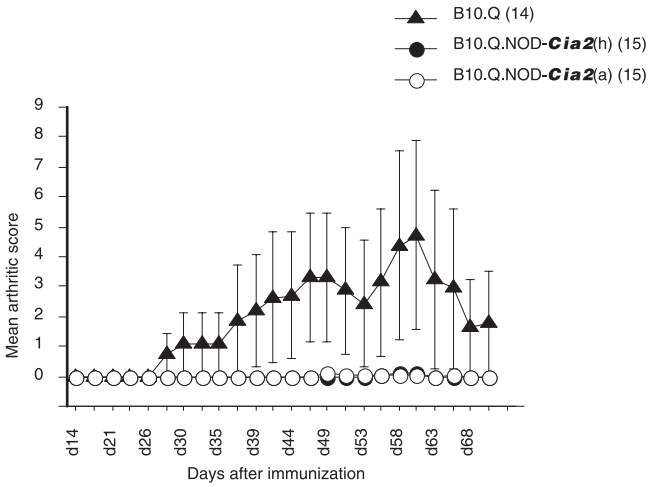

FIGURE 5. The *Cia2* locus dominantly suppress arthritis development in the B10.Q.NOD-*Cia2* congenic strain. The *Cia2* locus from NOD.Q on B10.Q genetic background dominantly suppresses the development of arthritis in the B10.Q.NOD-*Cia2* congenic strain. Arthritis severity expressed as mean maximum arthritic scores. The mice were investigated for arthritis for 70 days after collagen injection. B10.Q.NOD-*Cia2* (h), heterozygous *Cia2* congenics; B10.Q.NOD-*Cia2* (a), homozygous *Cia2* congenics.

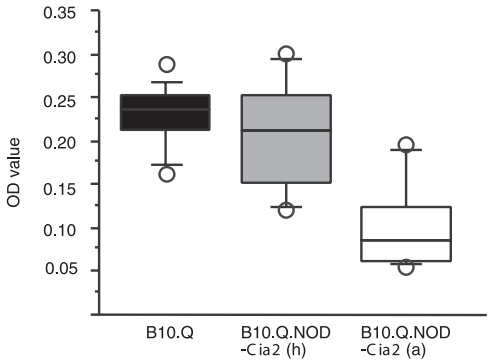

FIGURE 6. Levels of C5 in sera in B10.Q.NOD-*Cia2* congenic mice compared with B10.Q wild type. No detection of significant reduction of C5 levels in sera from mice heterozygosity of the *Cia2* locus from NOD.Q onto B10.compared with wild-type B10.Q. The levels of complement factor 5 in sera were detected using the ELISA in sera taken at day 35 after collagen II immunization. Levels in homozygous B10.Q.NOD-*Cia2* were used as background levels of the assay. B10.Q.NOD-*Cia2* (h), heterozygous; B10.Q.NOD-*Cia2* (a), homozygous.

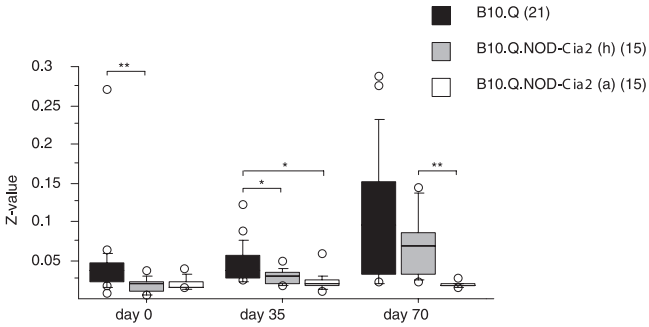

FIGURE 7. Reduced activity of alternative complement pathway after CIA induction in B10.Q.NOD-*Cia2* congenic mice illustrated by reduced hemolytic capacity. Activity of alternative pathway was measured as lysis of rabbit erythrocytes in sera taken from males mice at the day of collagen II immunization (day 0), and after 35 as well as 70 days, respectively. Z-value = lymph node (1 − percentage of lysis); Z-value = 1 when 60% of cells are lysed. Mean Z-values were compared using the Mann-Whitney *U* test, significance levels ∗, <0.05 and ∗∗, <0.005. n = number of mice per group B10.Q ($C5^{+/+}$) vs B10.Q.NOD-*Cia2* (h) ($C5^{+/-}$): $p_{d0} = 0.003$, $p_{d35} = 0.03$, $p_{d70} = 0.2$; B10.Q.NOD-*Cia2* (h) ($C5^{+/-}$) vs B10.Q.NOD-*Cia2* (a) ($C5^{-/-}$): $p_{d0} = 0.7$, $p_{d35} = 0.02$, $p_{d70} < 0.001$.

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 mice compared with wild type, there are detectable differences using a functional hemolytic assay. We conclude that the *Hc* gene plays the major role in the *Cia2* loci. However, it is important to emphasize that a major role for C5 in the *Cia2* still does not exclude a role for additional modifier genes. Such genes could only be determined through reducing the fragment in smaller pieces in subcongenic strains.

The role of complement in the development of arthritis is perhaps underestimated as a pathogenic factor that may differ between arthritis and diabetes. Complement activation can be observed in joint spaces of patients with RA, and it correlates with severity of the disease (35). Levels of C5a are high in synovial fluid of patients with RA (36). Further support for in vivo role of complement in arthritis is provided by the fact that mice lacking various complement components such as C5, C3, or factor B due to genetic manipulations do not develop arthritis (23, 37). Furthermore, monoclonal anti-C5 Abs are able to prevent CIA in mice (24) and are now subjected to clinical test in patients with RA. In addition to blocking of induction of the disease, these Abs block progression and partially reverse joint destruction in established disease. Although complement is one of the players in the development of arthritis, there are many additional ones. It is important to delineate the role of each complement component to rationally design 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.

Acknowledgments

We thank Inger Jonasson and Ann-Sofi Strand for help with the genotyping.

Disclosures

The authors have no financial conflict of interest.

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