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The Non-MHC Quantitative Trait Locus Cia5 Contains Three Major Arthritis Genes That Differentially Regulate Disease Severity, Pannus Formation, and Joint Damage in Collagen- and Pristane-Induced Arthritis

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Cia5 is a locus on rat chromosome 10 which regulates the severity of collagen- and pristane-induced arthritis (CIA and PIA). To refine the region toward positional identification, Cia5 subcongenic strains were generated and studied in CIA and CIA. The protective effect of the telomeric locus Cia5a was confirmed in both models. A second arthritis severity locus (Cia5d) was identified within the most centromeric portion of Cia5. DA.F344(Cia5d) rats had a significantly lower median arthritis severity index in PIA, but not in CIA, compared with DA. On histologic analyses DA.F344(Cia5a) and DA.F344(Cia5d) congenics with PIA preserved a nearly normal joint architecture compared with DA, including significant reduction in synovial hyperplasia, pannus, angiogenesis, inflammatory infiltration, bone and cartilage erosions. Cia5 and Cia5a synovial levels of IL-1β mRNA were reduced. Although both DA.F344(Cia5a) and DA.F344(Cia5a) rats were protected in CIA, the arthritis scores of DA.F344(Cia5a) were significantly higher than those of DA.F344(Cia5a), suggesting the existence of a third locus where F344-derived alleles centromeric from Cia5a contribute to increased arthritis severity. The existence of the third locus was further supported by higher levels of autoantibodies against rat type II collagen in DA.F344(Cia5a) congenics compared with DA.F344(Cia5a). Our results determined that Cia5 contains three major arthritis severity regulatory loci regulating central events in the pathogenesis of arthritis, and differentially influencing CIA and PIA. These loci are syntenic to regions on human chromosomes 17q and 5q implicated in the susceptibility to rheumatoid arthritis, suggesting that the identification of these genes will be relevant to human disease. The Journal of Immunology, 2005, 174: 7894–7903.

Rheumatoid arthritis (RA) is a chronic autoimmune disease with an overall prevalence of 0.5–1% in most populations. RA has a strong genetic component (1, 2) and several MHC and non-MHC susceptibility loci have been identified in family-based linkage studies (3–8). Recent studies based on positional cloning (9, 10) and candidate gene strategies (11, 12) have identified the first genes implicated in the susceptibility to RA. However, most susceptibility genes are yet to be identified. Additionally, very little is known about the genetic regulation of disease severity, in contrast to susceptibility (13, 14), and none of the available multi-institutional family-based genome-wide studies were specifically designed to address this issue. It has been considered that at least some of the susceptibility genes will have a more significant role in the regulation of early stages in disease pathogenesis, perhaps preclinical events (e.g., T cell responses), and have only a minor role in the regulation of established and chronic disease, stages that involve the recruitment of several components of the innate and acquired immune responses (15). Genes mostly regulating very early stages in RA pathogenesis may not make as good targets for the development of therapies or prognostication as those found to regulate established and chronic disease, as well as clinical disease severity. Therefore, we have been interested in the identification of arthritis severity regulatory genes. The identification of RA genes has been hampered by factors inherent to this complex trait such as variable penetrance, variable disease allele-associated relative risk, epistasis, genetic heterogeneity of human populations, and complex interactions between environmental and genetic factors (16). The study of rat models of arthritis in inbred strains that differ in their susceptibility to and severity of arthritis reduces several of these confounding variables (17, 18), thus facilitating the identification of loci regulating arthritis-related phenotypes, including disease severity (19–28), chronicity (23, 29, 30), as well as specific disease features, such as the production of rheumatoid factors (31). Once identified, these
rat arthritis genes will provide novel targets for focused candidate gene (or candidate pathway) analysis in RA case-control studies. Previous genome-wide screens have identified several quantitative trait loci (QTL) regulating disease severity in various rat models of RA (19–28). One of the identified QTLs, Clin5, was mapped to rat chromosome 10 in an intercross between the arthritis-susceptible DA and arthritis-resistant F344 rat strains studied for collagen-induced arthritis (ClIA). Studies in congenic rats confirmed the arthritis-regulatory effect associated with Clin5 and suggested that this QTL could be accounted for by at least two distinct genes (32). Moreover, this QTL was involved in the regulation of arthritis severity in collagen-, pristane-, oil- (33), and adjuvant-induced arthritis (34). Clin5 colocalizes with QTLs involved in the regulation of other models of autoimmune diseases (35–37) and its human syntenic regions contain loci regulating different forms of autoimmune diseases (discussed in Ref. 32), including RA (4, 38), suggesting that it harbors genes relevant not only to RA, but possibly to other diseases as well.

To localize and to narrow the intervals containing the two arthritis-regulatory genes, and to comprehensively characterize their regulatory effects in arthritis severity, joint histology, and synovial tissue cytokine gene expression, Clin5 subcongens were generated and studied for their susceptibility to and severity of pristane-induced arthritis (PIA) and ClIA, two well-established models of autoimmune erosive arthritis that share certain similarities in their pathogenesis and genetic regulation, but also have differences (23, 25, 33, 39, 40). We determined that Clin5 contains three different QTLs that regulate arthritis severity. In PIA, Clin5a and Clin5d also regulate synovial hyperplasia, pannus formation, angiogenesis, synovial inflammation, and bone and cartilage erosions, suggesting that these two genes control fundamental processes in the pathogenesis of arthritis. The novel QTL, Clin5d, is located in the more centromeric portion of Clin5, and regulates PIA only, while Clin5a regulates both PIA and ClIA and was reduced to 10 Mb. A third and novel locus specific for ClIA was identified in the region centromeric from Clin5a in which the arthritis severity favoring alleles come from F344. This third locus regulates levels of anti-collagen autoantibodies. The identification of the genes accounting for these three loci should increase our understanding of the processes regulating arthritis and point to novel candidate genes and pathways for studies in human disease.

Materials and Methods

Rats

Specific pathogen-free DA (DA/BklArb; arthritis-susceptible) and F344 (F344/Hsd, Harlan; arthritis-resistant) inbred rat strains were used in the breeding of the congenic and subcongenic strains. DA/BklArb were originally purchased from Bantin & Kingman, maintained at the Arthritis and Rheumatism Branch, NIAMS, NIH, and then transferred to the North Shore-LIJ Research Institute (NSLIJRI) (DA/BklArbhNSi) and used as controls. All the experiments involving animals were reviewed and approved by the NSLIJRI Institutional Animal Care and Use Committee.

Construction of the genotype-guided Clin5 QTL-congenic and subcongenic lines

An 89.7-Mb interval, containing the 15 Mb (16.5 cM) two logarithm of odds support interval comprising Clin5, was introgressed from F344 into the DA rats through eight backcrosses (BC8) followed by at least five intercrosses, as previously described (32). DA/F344(Clin5a) subcongens have been previously generated (32). The other subcongenic lines covering the Clin5 interval (see Fig. 1) were generated for the present study. DA/F344(Clin5a) congenics were backcrossed twice with DA rats, and then screened for new recombinants within the Clin5 interval (see simple sequence length polymorphism, markers used on Fig. 1). Offspring (BC10) heterozygous at similar recombinant segments were brother-sister mated, and their offspring (BC10F1) genotyped to ensure homozygosity of the expected intervals. Homozygous subcongens were used to expand the sub-

congenic lines. Experiments were done with offspring from second to fifth intercrosses (BC10F1). Homozygous DA/F344(Clin5a) rats were backcrossed with DA animals to generate DA/F344(Clin5a)BC10, rats, heterozygous at the Clin5a interval. These rats were also studied in PIA.

Genotyping

Tail tips were excised from 3- to 4-wk-old rats and DNA was extracted with the DNeasy kit (Qiagen). PCR conditions have been previously reported and were set up in 10-μl reactions (30, 32). Briefly, [32P]PCR products were resolved in acrylamide sequencing gels and fluorescent-based PCRs using a fluorescence-labeled forward primer were run in ABI 310 capillary genotypersequencers (Applied Biosystems). GENESCAN 3.1 software (Applied Biosystems) was used for data extraction and allele assignment of the fluorescent-labeled PCR products. All genotypes were manually checked by two readers and questionable readings rechecked or repeated. For marker details, see the Rat Genetic Database (www.niams.nih.gov/rtbc/ratbase/index.htm) and the Rat Genome Database (www.rgd.mcw.edu).

Induction of PIA

Eight- to 12-wk-old rats received 150 μl of pristane (2,6,10,14-tetramethyldodecanec; Sigma-Aldrich) by intradermal injection (day 0) (33, 39). The dose was divided between two injection sites at the base of the tail.

Induction of CIA

Bovine type II collagen (BII; Chondrex) was dissolved overnight in 0.1 N acetic acid at 4°C (2 mg/ml) and emulsified with IFA (Difco) to a final concentration of 1 mg/ml. Eight- to 12-wk-old rats were injected intradermally at the base of the tail with 2 mg/kg of BII divided into six injection sites (day 0), and a booster injection of 10 μg of BII/IFA administered on day 7 (20). Serum was obtained on day 18 and stored at −80°C until tested.

Arthritis scoring

We used a previously described arthritis scoring system (19–21, 30) that evaluates individual joints and measures arthritis severity according to joint size as follows: 1) interphalangeal, metacarpophalangeal, and metatarsophalangeal joints in each one of the four lateral digits were scored: 0, no arthritis; 1, arthritis present; 2, wrist, midforepaw, ankle and midfoot joints were scored; 0, normal; 1, minimal swelling; 2, moderate swelling; 3, severe swelling; 4, severe swelling and non-weight bearing. The scores from all involved joints were added (maximum score per rat = 80). The same observer obtained the arthritis scores and weights on days 0, 14, 18, 21, 24, 28, and 31 following induction. The arthritis severity index (ASI), which is a measure of disease severity over time (area under the curve), was determined for each animal by summing the individual arthritis scores obtained over the course of the experiment. The ASI was used because it provides a more comprehensive evaluation of arthritis severity.

Histology and histologic scoring

At the end of the arthritis observation period (day 32), the right hind paw was fixed in 10% formaldehyde. Paws were then decalcified with a solution containing hydrochloric acid and 0.1 M EDTA (Cal-Ex; Fisher Scientific). Tissues were sectioned, embedded in paraffin, and slides prepared and stained with H&E and safranin-O. Slides were scored without knowledge of strain identity. We used a recently described comprehensive histologic scoring system (41). Briefly, tibiotalar, taluscalcaneal, and midfoot joints were histologically scored for the following parameters:

Synovial inflammation. Five high-power magnification fields (HMF) were scored for the percentage of infiltrating mononuclear cells as follows: 0, absent; 1, mild (1–10%); 2, moderate (11–50%); 3, severe (51–100%). The mean of the five HMF was used for analyses.

Synovial hyperplasia. 0, absent; 1, mild (5–10 layers); 2, moderate (11–20 layers); 3, severe (>20 layers).

Extension of pannus formation based on the reader’s impression. 0, absent; 1, mild; 2, moderate; 3, severe.

Synovial fibrosis. 0, absent; 1, mild (1–10%); 2, moderate (11–50%); 3, severe (51–100%).

Synovial vascularity (angiogenesis). The number of vessels was counted in five HMF of synovial tissue and the mean used for analyses.

Cartilage erosion. Percentage of the cartilage surface that was eroded: 0, absent; 1, mild (1–10%); 2, moderate (11–50%); 3, severe (51–100%).

Cartilage degradation. Based on safranin-O staining of proteoglycans and described as the percentage of the cartilage that lost its staining: 0, none; 1, mild loss (1–10%); 2, moderate loss (11–50%); 3, severe loss (51–100%).
**Bone erosion.** 0, none; 1, minor erosion(s) observed only at HMF; 2, moderate erosion(s) observed at low magnification; 3, severe transcortical erosion(s).

**Quantitative real-time PCR (RT-PCR)**

Left ankle synovial tissue obtained after the completion of the arthritis observation period (day 32) was immediately frozen in liquid nitrogen. Tissues were subsequently homogenized and total RNA isolated with the RNeasy kit (Qiagen) and digested with DNase (Qiagen), according to the manufacturer’s protocol. Two micrograms of total RNA from each sample were used for cDNA synthesis (SuperScript II kit; Invitrogen Life Technologies). The quantitative real-time PCR method used was recently reported (41). Briefly, cDNA was optimized for relative gene expression by RT-PCR. The TaqMan 5’ exunuclease assay (Applied Biosystems) was used for the quantification of the rat TNF-α and IL-1β genes. GAPDH was used as endogenous control. TaqMan primers and probes were designed using Primer Express software, version 1.5 (Applied Biosystems; Ref. 41). Probes were labeled with FAM at the 5’ end and TAMRA at the 3’ end. Eurogentec qPCR mastermix reagents were used in an ABI 7700 Sequence Detection System (Applied Biosystems). The PCR mixture contained 1× mastermix, 500 nM of either TNF-α, IL-1β, or GAPDH forward and reverse primers, 200 nM TNF-α or IL-1β, or 100 nM GAPDH gene-specific TaqMan probes, and 5 μl of 1/20 diluted cDNA to a final volume of 25 μl. All samples were run in duplicates and the mean used for the analyses. Thermocycler conditions were 50°C for 2 min, 95°C for 10 min and 45 cycles of 95°C for 0.15 min and 60°C for 1 min. Data were analyzed using Sequence Detection System software version 1.9.1 (Applied Biosystems). Results were obtained as Ct (threshold cycle) values. The Ct value is inversely proportional to the starting template copy number. Relative expression of TNF-α and IL-1β in synovial tissues was adjusted for GAPDH in each sample (ΔCt). ΔCt values were compared with the Student’s t test. Fold-change differences in gene expression between DA, DA.F344(Cia5), and subcongens were compared with the 2^{ΔCt} method (Ref. 42 and ABI 7700 Sequence Detection System User Bulletin No. 2).

**Measurement of anti-collagen Abs**

Serum samples collected on day 18 after CIA induction were assayed for IgG Abs against BII and rat type II collagen (RII) using commercially available ELISA kits (Chondrex), according to the manufacturer’s instructions. The IgG Ab concentration in micrograms per milliliter was calculated using the equivalence ratio of 82 U/ml to 1 μg/ml.

**Statistical analyses**

Based on our previous observation of gender differences in the genetic regulation of CIA (32, 33), males and females were studied separately for their susceptibility to and severity of arthritis. Non-normally distributed data (medians) were compared with ANOVA on ranks with a pairwise multiple comparison procedure (Dunn’s method) for multiple groups, or with the Mann-Whitney rank sum test for two group comparisons. The Student t test was used to compare normally distributed data (gene expression). A p value of 0.05 or less was regarded as significant. All statistical analyses were done with SigmaStat 3.0 (SPSS).

**Results**

DA.F344(Cia5) congenics, and Cia5a, Cia5e, and Cia5d subcongens were protected and developed a significantly milder form of PIA

The introgression of the F344-derived Cia5-containing interval into the DA background, as in the DA.F344(Cia5) congenics (Fig. 1), was associated with a highly significant reduction of 97 and 98% in median ASI in male and female congenics, respectively, as compared with same sex DA rats (Fig. 2A) (median ASI: male DA = 127, male DA.F344(Cia5) = 4, p ≤ 0.001; female DA = 114, female DA.F344(Cia5) = 2, p ≤ 0.001). This protective effect could be noticed at day 14 (Fig. 2A) and persisted throughout the observation period, suggesting that the arthritis gene located...
FIGURE 2. Arthritis severity clinical scores (mean ± SEM) in DA and congenic rats during a 31-day observation period. Squares, males; diamond, females; black, DA; white, congenic strain. PIA: A, DA and DA.F344(Cia5); B, DA and DA.F344(Cia5a); C, DA and DA.F344(Cia5b); D, DA and DA.F344(Cia5c); E, DA and DA.F344(Cia5d). CIA: F, DA and DA.F344(Cia5); G, DA and DA.F344(Cia5a); H, DA and DA.F344(Cia5b); I, DA and DA.F344(Cia5e); J, DA and DA.F344(Cia5d).
within Cia5 regulates both early/induction and chronic/perpetuation stages during the course of disease pathogenesis.

Male and female rats subcongenic for the most telomeric 16-Mb interval of Cia5, DA.F344(Cia5a) (Fig. 1) also had significantly lower arthritis scores (Fig. 2B), with a decrease in median ASI of 95 and 89%, respectively, compared with DA (median ASI: male DA = 127, male DA.F344(Cia5a) = 6.5, \( p = 0.001 \); female DA = 114, female DA.F344(Cia5a) = 12, \( p = 0.001 \)). To determine the mode of inheritance of Cia5a, DA.F344(Cia5a) rats were backcrossed with DA and males (BC1) heterozygous at the Cia5a interval and studied in PIA experiments. Cia5a heterozygous (het) rats developed similar ASI as those of Cia5a homozygous congenics (\( p = 0.064 \)) and significantly lower ASI compared with DA (median male ASI: DA = 127, DA.F344(Cia5a)het = 14.5; \( p = 0.001 \)) (data not shown), suggesting that the DA-originated arthritis severity alleles operate in a recessive manner.

Both male and female DA.F344(Cia5e) subcongenics (60.57-Mb interval; Fig. 1) had significantly lower PIA scores compared with same sex DA (Figs. 2D and 3B), with reductions in median ASI of 69 and 76%, respectively (median ASI: male DA = 127, male DA.F344(Cia5e) = 39, \( p = 0.001 \); female DA = 114, female DA.F344(Cia5e) = 27, \( p = 0.001 \)). Rats subcongenic for the most centromeric 41-Mb segment (47.3 Mb including the regions where recombinations took place) of Cia5, DA.F344(Cia5d) (Fig. 1), were also protected from PIA (Fig. 2C), with reductions in median ASI of 46% for males and 55% for females, compared with same sex DA controls (Fig. 3C) (Median ASI: male DA = 127, male DA.F344(Cia5d) = 69, \( p = 0.018 \); female DA = 114, female DA.F344(Cia5d) = 51, \( p = 0.024 \)). These findings suggested that the same gene accounts for Cia5d and Cia5e, and that this gene is located within the interval shared by these two subcongenics.

DA.F344(Cia5b) subcongenics (Fig. 1) were not protected (Fig. 2C) and developed a similar ASI as same sex DA rats (Figs. 2C and 3D) (median ASI: male DA = 127, male DA.F344(Cia5b) = 86.5, \( p = 0.28 \); female DA = 114, female DA.F344(Cia5b) = 110, \( p = 0.38 \)), demonstrating that this interval did not contain an arthritis gene. The median DA.F344(Cia5b) ASI was significantly higher than that of DA.F344(Cia5) (males and females, \( p = 0.01 \)), DA.F344(Cia5a) (males and females, \( p = 0.01 \)), and DA.F344(Cia5e) (males, \( p = 0.015 \); females \( p = 0.007 \)). DA.F344(Cia5a) and DA.F344(Cia5b) rats overlap part of their congenic interval (Fig. 1), and based on the phenotypic differences we were able to exclude that common region as the one containing the arthritis gene, thus narrowing the Cia5a interval to 10.1 Mb. Taken together, these data demonstrate that Cia5 contains two PIA-regulatory genes that influence disease expression both in males and females. One gene is located within the telomeric portion of the Cia5a interval that does not overlap Cia5b and the other is within the Cia5d and Cia5e shared centromeric interval.

DA.F344(Cia5d) congenics and DA.F344(Cia5a) subcongenics developed a significantly milder form of CIA

To determine whether the same intervals regulating PIA also regulated CIA, Cia5 congenics and subcongenics were studied in this model. Both DA.F344(Cia5) males and females tested for CIA were protected (Fig. 2F) and had lower median ASI (36 and 53%, respectively) than same sex DA rats (median ASI: male DA = 250, male DA.F344(Cia5) = 160, \( p = 0.01 \); female DA = 330, female DA.F344(Cia5) = 154.5, \( p = 0.01 \)). DA.F344(Cia5a) males and females also displayed significantly lower median ASI than DA (92 and 98%, respectively) (Fig. 2G) (median ASI: male DA = 250, male DA.F344(Cia5a) = 20, \( p = 0.001 \); female DA = 330, female DA.F344(Cia5a) = 5, \( p = 0.001 \)). Although the ASI of DA.F344(Cia5) congenics were significantly lower than those of DA, they were significantly higher than those of DA.F344(Cia5a) both in males and females (\( p = 0.004 \)), suggesting that F344-derived alleles at a novel locus centromeric from Cia5a but within Cia5 contribute to increased disease severity. The ASI of DA.F344(Cia5b), DA.F344(Cia5e), and DA.F344(Cia5d) subcongenics were not significantly different than same sex DA rats (Fig. 2, H–J). Taken together, these data show that Cia5 contains two distinct CIA-regulatory genes, one contained in the Cia5a interval also regulating PIA, and in which the arthritis severity favoring allele originates from DA. The other gene is contained within the Cia5e interval, centromeric from Cia5a, but unlike the Cia5d CIA-regulatory effect, the CIA regulatory allele originates from F344, suggesting that this is a different gene. DA.F344(Cia5d) rats were not protected in CIA. No gender-specific effect or differences were observed.

Histologic studies in PIA synovial tissues

Histologic findings did not differ between males and females within each strain. Therefore, data from both genders were combined for analyses. Joint histologic architecture was highly abnormal in DA rats with PIA (Table I; Fig. 4A), with pronounced synovial hyperplasia and pannus formation (Fig. 4C), increased number of synovial vessels (angiogenesis) (Fig. 4D), synovial infiltration with mononuclear cells (Fig. 4E), and extensive cartilage and bone erosive changes (Figs. 4, A and G), and cartilage loss of proteoglycans (Fig. 4G). However, both DA.F344(Cia5) (Fig. 4B), DA.F344(Cia5a), and DA.F344(Cia5d) (Fig. 4F) preserved a nearly normal joint histologic architecture (Table I). No synovial hyperplasia was observed in DA.F344(Cia5), DA.F344(Cia5a), and only mild focal synovial hyperplasia was observed in a few of the DA.F344(Cia5d) ankles (Fig. 4F). DA.F344(Cia5), DA.F344(Cia5a), DA.F344(Cia5d), and DA.F344(Cia5d) had a significantly lower number of synovial vessels and synovial-infiltrating mononuclear cells, and diminished cartilage and bone erosive changes (Fig. 4, B,
F, and H). These histologic findings further support the observation of reduced clinical arthritis severity, and suggest that the Cia5a and Cia5d genes regulate processes central to the pathogenesis of synovial hyperplasia, pannus formation, and joint destruction in arthritis.

**Synovial tissue levels of IL-1β mRNA were significantly reduced in DA.F344(Cia5) and DA.F344(Cia5a), compared with DA rats**

Quantitative RT-PCR analysis revealed a reduction of >50% in the levels of IL-1β in the synovial tissues of DA.F344(Cia5) and DA.F344(Cia5a) rats obtained at day 32, compared with DA (ΔCt: DA = 8.09, Cia5 = 9.33 (p ≤ 0.01), Cia5a = 9.34 (p = 0.02), Cia5d = 9.15 (p = 0.14); the ΔCt is inversely correlated with the number of copies of mRNA in the tissue) (Fig. 5). IL-1β levels in Cia5d also tended to be lower than DA, however that difference did not reach statistical significance. TNF-α expression showed a similar trend toward reduced expression in Cia5 congenics, but lower than 50% (ΔCt: DA = 10.58, Cia5 = 11.43 (p = 0.09), Cia5a = 11.28 (p = 0.20), Cia5d = 11.13 (p = 0.46)). The mild differences in TNF-α expression between congenics and DA may be attributed to the use of day 32 tissues, a time point beyond the peak of synovial TNF-α expression.

**Reduced levels of Abs against BII and RII in Cia5a subcongenic strains tested for CIA**

High levels of IgG Abs against BII were detected in all strains immunized with BII/IFA, confirming appropriate immunization (Fig. 6A). There was good concordance between median levels of anti-BII and median levels of autoantibodies anti-RII in each strain. Levels of anti-BII and anti-RII were significantly lower in Cia5a and reduced compared with all other tested congenics, particularly in the case of anti-RII (Fig. 6). Levels of anti-BII and anti-RII tended to be higher in Cia5 compared with all other strains, including DA. Levels of anti-BII and anti-RII Abs in DA rats were not significantly different from those in Cia5, Cia5b, Cia5d, and Cia5e. The concordance between arthritis severity scores and Ab levels in Cia5a suggests that the magnitude of the Ab response against the immunogen or the self-Ag may be involved in the regulation of disease severity. Additionally, the trend toward having higher levels of anti-collagen Abs in Cia5a rats, compared with both Cia5a and DA, further supports the concept outlined above that F344-derived alleles centromeric from Cia5a contribute to increasing disease severity. Interestingly, Cia5e subcongens, which contain F344 alleles through the whole Cia5a interval centromeric from Cia5a, did not produce anti-RII levels similar to Cia5, suggesting that the interaction (epistasis) between F344 alleles at Cia5a and at Cia5e is required for the increased levels of anti-RII.

**Discussion**

RA is a chronic disease associated with pain, difficulty executing daily activities, increased risk for disability, reduced survival, and reduced personal income (15). New biologic therapeutic agents have greatly contributed to improved disease control (43–46), yet remission is rarely achieved. Therefore, the identification of genes regulating RA has the potential to increase our understanding of disease pathogenesis and also to identify novel pathways and molecular targets for the development of new and perhaps more effective treatments. Although several ongoing familial-based genome-wide studies are aiming to identify RA susceptibility genes, none of them was specifically designed to look for genes regulating disease severity. We consider that some of the susceptibility genes may only regulate very early processes in disease pathogenesis and not necessarily the processes controlling established/chronic disease. Because arthritis severity genes regulate disease expression during established/chronic stages, we envision that those genes are more likely to become useful targets for the development of therapies and prognostication tools.

To identify arthritis severity regulatory genes, we have conducted genetic analyses in rat intercrosses generated between arthritis-susceptible and -resistant strains studied in different models of autoimmune arthritis (19–22, 30). The chromosome 10 QTL Cia5 was originally identified in a DA × F344 F₂ intercross studied for CIA (20), and its arthritis regulatory effect confirmed in DA.F344(Cia5) congenics (32). Our previous study focused on clinical evaluation of arthritis severity and suggested the possibility that the Cia5 congenic interval contains at least two CIA and PIA regulatory loci, one of which is located within the Cia5a interval (33). In the present study, we report the generation of Cia5 subcongenic strains and the identification and localization of two novel arthritis-regulatory loci, Cia5d, a PIA-regulatory locus within the most centromeric 41.5-Mb interval (47 Mb including the recombination regions) of Cia5, and another locus contained within the Cia5e interval, centromeric from Cia5a, in which F344-derived alleles specifically increase disease severity in CIA. We also confirmed the arthritis-regulatory effect of Cia5a and reduced the interval within which it is located to 10 Mb.

In addition to evaluating clinical arthritis severity during a 31-day observation period, comprehensive histologic analyses that evaluated several different parameters and the quantification of synovial IL-1β and TNF-α mRNA were conducted. These analyses revealed that, in addition to reducing PIA clinical arthritis severity,
the presence of F344 alleles at either Cia5a or Cia5d was associated with preservation of the joint architecture, significant reduction in synovial hyperplasia and pannus formation, reduction of the synovial inflammatory infiltration, and number of synovial vessels (angiogenesis). Additionally, Cia5a and Cia5d congenic strains had significant reduction in cartilage and bone erosive changes and both had reduced synovial levels of IL-1β message even when tissues were obtained at a later time point during disease course (day 32), compared with DA. These findings demonstrate that the regulatory effects of Cia5a or Cia5d persist beyond the early stages of disease and into the chronic/perpetuation stages. The synovial events affected by the presence of the Cia5a or Cia5d intervals have been previously shown to be at least in part interdependent. Specifically, angiogenesis is a critical event in the pathogenesis of autoimmune arthritis and in the development of synovial hyperplasia (47, 48). Similarly, synovial hyperplasia and pannus formation are typically associated with cartilage and bone erosions in arthritis (41, 49), and are partially dependent on the inflammatory mononuclear cellular infiltrate and the cytokines these infiltrating cells produce (50–53). Moreover, several of the cytokines produced by the synovial tissues and infiltrating mononuclear cells have angiogenic properties (54). Therefore, while we cannot determine at this point which specific cellular and molecular events the Cia5a and Cia5d genes regulate, our observations demonstrate that these genes control fundamental events required for the development of synovial inflammation, arthritis, and articular damage. It is conceivable that these two genes will be key regulators of arthritis pathophysologic events, similar to what has been described using NF-κB (50).

The presence of F344 alleles within the Cia5a interval was associated with significant reduction in arthritis scores both in PIA and CIA, suggesting that the arthritis gene contained within this interval regulates a cellular and molecular process critical for both models. Cia5a congenics had lower levels of anti-BII and autoantibodies to anti-RII, raising the possibility that this gene influences the arthritis phenotype via the regulation of B cell functions. Furthermore, our observation that the protective effects of the Cia5a allele were similar in DA.F344(Cia5a) heterozygotes and DA.F344(Cia5d) homozygotes indicates that the Cia5a allele acts recessively to confer increased arthritis severity. Knowledge of the mode of inheritance of the Cia5a gene will greatly facilitate the identification effort because heterozygous recombinant backcross offspring can be used in arthritis studies without having to further intercross recombinants to generate homozygosity. Cia5d congenic rats were only protected in PIA, and not in CIA, suggesting that this gene regulates a pathway critical for PIA and not for CIA. This observation is in agreement with the original localization of the Cia5 locus in the DA × F344 F2 genome-wide screen (20). However, a genome-wide screen done in a BB × BN F2 intercross studied for CIA identified a Cia5d-colocalizing locus, Cia16, further suggesting that this interval contains an arthritis-regulatory gene that is not limited to a single disease model. Moreover, one cannot completely exclude the possibility that Cia5d could interact with yet another locus in an epistatic manner in the regulation of CIA in DA × F344 congenics. Because Cia5d directly and significantly regulated PIA, this will be the model of choice in the process of narrowing down the critical region toward the identification of the specific gene.

Unexpectedly, Cia5 had significantly higher CIA arthritis severity scores than Cia5a, suggesting the possibility of a third regulatory locus located in the region centromeric from Cia5a (within...
Levels were determined by ELISA and analyzed with ANOVA on ranks with a pairwise multiple comparison procedure (Dunn’s method). A p value of <0.05 was regarded as significant. Ab levels in DA compared with Cia5a subcongens. Ab levels in DA were not significantly different from Cia5, Cia5b, Cia5d, and Cia5e congenics. Cia5 rats tended to have higher levels of Ab against type II collagen than any other strain tested.

Our previous studies had suggested that Cia5a operated in a female-specific manner to regulate CIA severity (32). In the present study, both male and female DA.F344(Cia5a) congenics were significantly protected and developed a milder form of disease. Three possible explanations could account for these differences. First, the present study was conducted at the NSLIJR, while the previous study was conducted at the NIH. Environmental differences between the two institutions, including the rat intestinal flora, could have influenced immune responses. Second, there were certain differences in reagents that could have affected the resulting immune responses. Specifically, the source of type II collagen was different, and while the same source of IFA was used, it is conceivable that the adjuvant effect could vary from the lot used at the NIH to the one used at the NSLIJR. The male and female arthritis protection seen in the present study in association with the Cia5a interval in fact suggests a potentially broader relevance of our findings from rat studies will be relevant to RA and may identify novel genes for focused case-control testing.

Cia5a, and containing an F344-derived arthritis severity-favoring allele. This possibility was further supported by the higher levels of anti-BII and anti-RIL observed in Cia5 congenics as compared with Cia5a. However, the arthritis severity and Ab levels in Cia5e were not different from DA, suggesting that the CIA arthritis-favoring effect associated with F344 alleles at Cia5e depend on the presence of F344 alleles at Cia5a (epistasis). Another possibility is that in the absence of F344 alleles at Cia5a the arthritis severity is so close to maximum that it leaves little room for the detection of any additional contributions to severity. To confirm and better resolve the location of this putative locus, Cia5e and Cia5d subcongens will be bred with Cia5a to generate double congenics in an attempt to recreate the Cia5 phenotypes.

The three arthritis regulatory intervals studied herein colocalize with loci regulating different forms of autoimmune arthritis in rats (19, 27, 58). Alternatively, Cia5d colocalizes with a murine arthritis locus Pgia7 (59), and most importantly, it contains SLC22A4 (human chromosome 5q23.3) (11), a gene recently implicated in the susceptibility to RA in Japan. Although candidate gene analyses are typically fruitless when dealing with a genomic interval as long as Cia5d, the rat homologue of SLC22A4, Ocm1, was a compelling candidate. Preliminary cDNA sequencing analyses did not reveal any differences between the coding regions of DA and F344 Ocm1 (M. Brenner and P. Gulko, unpublished observations). Additional candidate genes of interest in the Cia5d interval include IL-3, IL-4, IL-5, and TIM-1.

The Cia5a syntenic region on human chromosome 17q22-q25 contains two RA susceptibility loci recently identified in genomewide scans (4, 38). Taken together, the colocalization of multiple arthritis regulatory loci and genes to the same, or syntenic, interval across different species, including humans, suggests that the findings from rat studies will be relevant to RA and may identify novel genes for focused case-control testing.

Cia5a and Cia5d also colocalize with several loci, or their syntenic regions, implicated in the regulation of rodent models of autoimmunity and inflammation (35–37, 60–62), or in the susceptibility to human autoimmune diseases (63–66). These studies suggest that both Cia5a and Cia5d genes could be relevant not only to arthritis, but also to other autoimmune diseases. The concept of colocalization of non-MHC loci regulating different forms of autoimmune diseases has been previously proposed (67, and recently confirmed with the discovery that polymorphisms in CTLA4 (68) and PTPN22 (12, 69) regulate more than one type of autoimmune disease. We consider that Cia5a and Cia5d could
become additional examples of such autoimmune regulatory genes common to more than one autoimmune disease.

In conclusion, the present study described two novel arthritis-susceptibility genes: a replication study and combined analysis of a genomewide linkage study of rheumatoid arthritis, including covariates. Arthritis Rheum. 50: 2757–2765.

Acknowledgments

We thank Dr. Franak Batiwalla and Houman Khalili, and Dr. Alamelu Chandrasekaran for their technical assistance with the ELISAs and quantitative PCRs, respectively.

Disclosures

The authors have no financial conflict of interest.

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