

# Identification and Isolation of Dominant Susceptibility Loci for Pristane-Induced Arthritis<sup>1</sup>

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Rheumatoid arthritis is a chronic inflammatory autoimmune disorder, controlled by multiple genes as well as environmental factors. With animal models, like the pristane-induced arthritis (PIA) in rats, it is possible to reduce the environmental effects and the genetic heterogeneity to identify chromosomal regions harboring genes responsible for the arthritis development. The PIA model has proved to be useful for identifying gene regions controlling different phases of the disease based on intercrosses between the resistant E3 and the susceptible DA rat. We have now performed a high-powered backcross analysis that confirms previous intercross-based data but also identifies additional loci. Earlier identified PIA loci were reproduced with high significance; *Pia1* (MHC region on chromosome 20), *Pia4* (chromosome 12), and *Pia7* (chromosome 4) are all major regulators of PIA severity and were also found to operate in concert. These three loci were verified in congenic strains using both disease- and arthritis-inflammatory-related subphenotypes as traits. We were also able to detect five new quantitative trait loci with dominant effects on PIA: *Pia10*, *Pia12*, *Pia13*, *Pia14*, and *Pia15* on chromosomes 10, 6, 7, 8, and 18, respectively. These data highlight the usefulness of the statistical power obtained in a backcross of a complex disease like arthritis. *The Journal of Immunology*, 2003, 171: 407–416.

Rheumatoid arthritis (RA)<sup>4</sup> primarily affects peripheral joints where a chronic inflammatory synovitis often results in cartilage destruction, bone erosion, and ultimately, joint deformity and loss of function. The pathogenesis of RA is poorly understood, and the diagnosis is based on clinical descriptions rather than on an understanding of the disease mechanisms (1). Adding to the complexity is the fact that RA is influenced by both the environment (2) and genetics (3).

Evidence for a genetic contribution to RA has been produced using twin association studies (4, 5), in which the concordance rate for monozygotic twins was determined to be ~15%, although a refined analysis using the same material came to the conclusion that an inherited liability to RA could be as high as 60% (6). Although once postulated to be dominant Mendelian disorders (7), most autoimmune diseases, like RA, are complex polygenic diseases that to some extent share a common genetic predisposition (8, 9). Extensive efforts have been put into linkage and association studies of large human RA patient materials. However, polygenic diseases like RA do not follow simple inheritance patterns, but are complicated by genetic heterogeneity and genetic as well as environmental interactions (10). As a result, no linkages or associations to regions, outside the MHC, have been established in studies of

human RA (11, 12). In fact, the association with the MHC region, as is the case for most other autoimmune diseases, has been estimated to account for only about one-third of the genetic risk (13), leaving the major genetic component(s) unidentified. Furthermore, linkage analysis in humans is hampered by the need for a considerable number of families or carefully matched case and control groups to reach significance. Hence, the inability to control the environment and the difficulties in confirming and identifying the disease-regulating genes make association and linkage studies of polygenic diseases in humans elusive.

Animal models of RA have the advantages that the animals, often rodents, can be housed in a controlled environment and that the family material can be extended to identify and isolate candidate genes of an identified quantitative trait locus (QTL) (14).

We have used rat models with striking similarities to human RA (15) in a system to examine the genetics of arthritis. Rat models that fulfil the criteria used for diagnosis of RA in humans include those with cartilage-restricted Ag-induced arthritis, such as collagen type II-induced arthritis (16), and pristane (2,6,10,14-tetramethylpentadecane)-induced arthritis (PIA) (17). Several publications using these models reveal highly significant linkages to overlapping and unique chromosomal regions (18–27), demonstrating that these complex diseases are determined by far fewer genes than previously postulated for quantitative traits (28). In the cumulated work on arthritis models in rats, several QTLs have been isolated and confirmed in congenic strains (29–31), but until now, only the *Pia4* gene on rat chromosome 12 (27) has been positionally identified (32).

In a congenic strain, the chromosomal region harboring the identified QTL from the resistant strain replaces the corresponding region in the susceptible strain or vice versa (33). As the work to produce congenic strains is both time consuming and expensive, efforts to identify genes regulating polygenic diseases should focus on QTLs with high penetrance and preferably dominant effects. In most linkage analyses of arthritis in rats, F<sub>2</sub> intercrosses have been used as a base for the linkage analysis. This is the most optimal cross to obtain a general picture of the QTLs segregating between the two parental strains and to get an estimate of their additive and dominance effects. However, a backcross analysis will reduce the

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<sup>4</sup> Abbreviations used in this paper: RA, rheumatoid arthritis; QTL, quantitative trait locus; PIA, pristane-induced arthritis; COMP, cartilage oligomeric matrix protein; AGP,  $\alpha$ 1-acid glycoprotein; LOD, logarithm of likelihood.

significance threshold, leading primarily to the detection of dominant loci with higher significance (34).

We have previously reported genetic segregation analyses of PIA-susceptible DA and resistant E3 rats, showing strong linkages between arthritis phenotypes and several different chromosomal regions (24, 25, 27). In all of these projects, intercross experiments segregating the genomes of E3 and DA rats were analyzed. Using these intercross experiments, several susceptibility loci were identified (i.e., *Pial* to -8). In this study, we used a backcross strategy to increase the power of QTL detection as well as to highlight the most prominent candidate QTLs for congenic strain verification and future positional cloning. In addition to the use of clinical arthritis as the phenotype, we also investigated subphenotypes like cartilage destruction (plasma cartilage oligomeric matrix protein (COMP)) (35), acute inflammatory response ( $\alpha_1$ -acid glycoprotein (AGP)) (25), and alteration in CD4:CD8 cell ratio (our unpublished observations) to detect major arthritis-regulating loci. By using this backcross approach, we confirmed the previously located QTLs, *Pial* (chromosome 20), *Pia4* (chromosome 12), and *Pia7* (chromosome 4) with highly significant logarithm of likelihood (LOD) scores. We also isolated and reproduced these QTLs in congenic strains, using both disease and subphenotypes as traits. We also identified five new arthritis-controlling QTLs denoted *Pia10* and -12 to -15 on chromosomes 10, 6, 7, 8, and 18, respectively.

## Materials and Methods

### Animals

Pathogen-free rats of the E3 and DA strains (originating from Zentralinstitut für Versuchstierzucht, Hannover, Germany) were kept in animal facilities in a climate-controlled environment with 12 h light/dark cycles, housed in polystyrene cages containing wood shavings, and fed standard rodent chow and water ad libitum. The rats were found to be free from common pathogens including Sendai virus, Hantaan virus, coronavirus, reovirus, CMV, and *Mycoplasma pulmonis*. Female E3 and male DA rats were intercrossed to produce (E3 × DA)<sub>F1</sub> offspring that were further backcrossed to female DA rats to produce 650 DA(E3 × DA) rats.

DA.Pia4 (D12 Rat28 to D12 Mgh3; N10) and DA.Pia7 (D4 Mit16 to D4 Mgh11; N7) congenic rats (a, E3 allele; b, DA allele) were obtained through conventional backcross breeding to parental DA rats with negative selection of all known PIA QTLs and positive selection using microsatellite markers on chromosome 12 or chromosome 4, respectively. DA. *Pial* congenic rats were produced through marker-assisted breeding and were verified as a pure congenic line after six generations of backcross breeding to DA.

### Induction and evaluation of arthritis

Arthritis was induced by an intradermal injection at the base of the tail with 150  $\mu$ l of pristane (2,6,10,14-tetramethylpentadecane; Aldrich, Milwaukee, WI). Arthritis was induced in all rats at the age of 8–12 wk. Arthritis development was monitored by a macroscopic scoring system of the four limbs ranging from 0 to 15 (1 point for each swollen or red toe, 1 point for midfoot digit and knuckle, and 5 points for a swollen ankle). The scores of the four paws were added, yielding a maximum total score of 60 for each rat. The rats were observed one to four times per week for 28 days after pristane injection. At day 28, the swelling of the hind paws was determined in millimeters, using a caliper. At day 28, blood was obtained by cutting the tip of the tail. To prevent blood coagulation, 10  $\mu$ l of heparin (5000 IU/ml; Lövens Läkemedel, Malmö, Sweden) were mixed with 500–1000  $\mu$ l of blood. The plasma was separated from blood cells by centrifugation and stored at -20°C until assayed. After plasma collection at day 28, the rats were sacrificed and the spleen was surgically removed and used for analysis of the CD4:CD8 ratio.

### Determination of plasma protein concentrations

Levels of  $\alpha_1$ -AGP were measured with a soluble competitive radioimmunoassay (36) using rat  $\alpha_1$ -AGP (Zivic Laboratories, Porterville, PA) and a polyclonal rabbit Ab against rat  $\alpha_1$ -AGP (Agriseria, Vännäs, Sweden). Plasma concentration of COMP was determined by a competitive ELISA (37). Rat COMP was used for coating of the microtiter plates and for preparing the standard curve included in each plate. Plasma COMP was

detected by using a polyclonal antiserum raised against rat COMP (generously provided by Prof. D. Heinegård, Lund, Sweden) as capturing Ab.

### FACS analysis of CD4:CD8 ratio

Spleens were removed at day 28 post-arthritis induction, homogenized, and hemolysed using ammonium chloride (0.84%; pH 7.4). Cells were resuspended in PBS supplemented with BSA (0.5% w/v) and sodium azide (0.01% w/v). The following mouse Abs used for staining were purchased from BD Pharmingen (San Diego, CA): anti-CD4-FITC (OX-35), anti- $\alpha$ BTCT-PE (R73), and anti-CD8a-biotin (OX-8). Streptavidin-allophycocyanin was used as a secondary reagent. Propidium iodine was added before acquisition. Ten thousand cells were acquired for each sample, and gates were set to include all viable  $\alpha$ BT cells and analyzed for CD4:CD8 ratios, using a FACScan (BD Biosciences, Mountain View, CA) and CellQuest software (BD Biosciences).

### Genotyping and linkage analysis

DNA was prepared from toe biopsies by heating the sample in 1 ml of 50 mM NaOH for 1 h (38). The DNA solution was neutralized with 100  $\mu$ l of 1 M Tris buffer and used directly in PCR. Primer sequences for rat microsatellite markers defined as *DxMity*, *DxMghy*, *DxRaty*, and *DxGoty* were obtained from Research Genetics (Huntsville, AL), and those for markers defined as *DxWoxy* were obtained from the Wellcome Institute for Human Genetics (Oxford, U.K.). All markers were assayed by PCR on PTC-200 Thermal Cycler (MJ Research, Waltham, MA) according to the standard protocol. Resulting PCR products were run on ABI 377 DNA sequencer (PerkinElmer, Emeryville, CA) or MegaBACE 1000 (Amersham Pharmacia Biotech, Uppsala, Sweden), and data were analyzed with the software packages GeneScan 3.1 and Genotyper 2.1 (PerkinElmer) or Genetic Profiler 1.1, respectively, through comparison with amplified samples from parental strain rats.

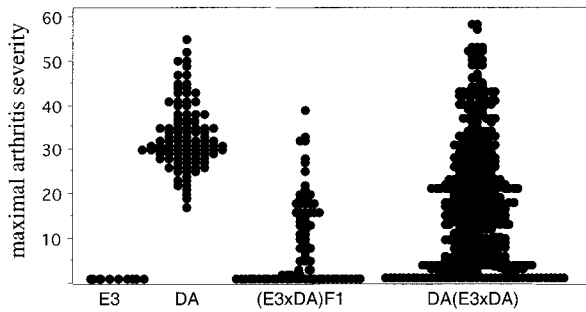
To produce linkage maps covering the complete genome, all 650 of the backcross progeny were genotyped using 236 markers, resulting in a dense map with an average distance of  $6.8 \pm 5.0$  cM and maximal intramarker distance of 19.8 cM. All autosomal chromosomes were analyzed, whereas the analysis of the X chromosome was excluded due to the composition of the breeding in which male (E3 × DA)<sub>F1</sub> rats were used in a backcross with female DA rats. An improved linkage map based on several crosses involving E3 and DA can be found at <http://net.inflam.lu.se> together with the map of the markers used in the present analysis.

Map Manager QTXb17 software (39) was used to perform QTL analysis and permutation tests and to draw chromosomal QTL maps showing the LOD of QTL controlling arthritis or arthritis-related phenotypes. The significance threshold values were obtained from permutation tests performed by randomizing the phenotypes 1000 times against the genotypes, because permutation calculations based on the investigated material validates a more accurate estimation of significance levels (40). The threshold values of the permutation test, which are labeled significant and highly significant, are derived from the guidelines of Lander and Kruglyak (41) and correspond to the thresholds representing  $\alpha = 0.001$  for a complete genome scan. Permutation analysis is used to determine significance levels based on the analyzed sample material. This is performed by randomizing the phenotypes >1000 times against the genotypes to calculate relevant significance levels. The same method was used by Lander and Kruglyak (41), but on material that was totally computerized and randomized. Therefore, permutation calculations based on the investigated material give a more accurate estimation of significance levels. No correction of significance for multiple testing was performed, because the degree of dependency between the different traits is hard to evaluate. All phenotype traits were transformed using natural logarithm to normalize an otherwise-not-normal distribution of analyzed data. Quantitative data are expressed as mean  $\pm$  SEM; significance analysis on congenic strains was performed using the nonparametric Mann-Whitney *U* test or, in the case of frequency, by  $\chi^2$  analyses.

## Results

### Linkage analysis

DA rats are 100% susceptible to PIA with a severe disease and a highly reproducible time of onset, whereas E3 rats, being totally resistant, represent the other extreme. In the *F1* generation, an intermediate arthritis phenotype is observed, whereas the gene-segregating DA(E3 × DA) backcross shows a broader spectrum of arthritis susceptibility and severity (Fig. 1, Table I). This disease distribution clearly argues against a single dominant gene. Linkage



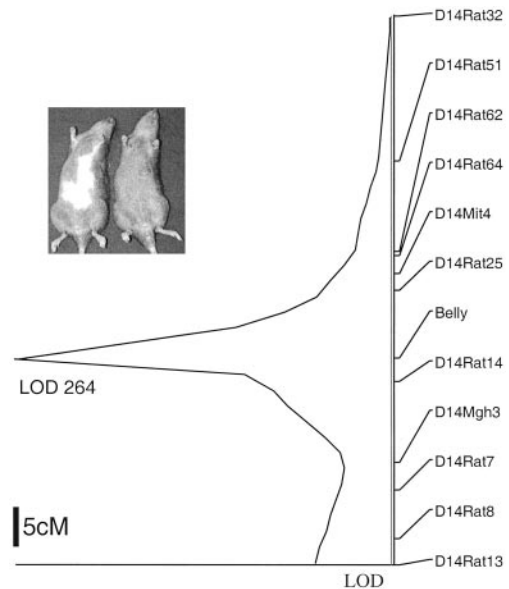
**FIGURE 1.** Distribution of maximal clinical arthritis after pristane injection for E3 and DA parental strains and (E3 × DA)<sub>F1</sub> and DA(E3 × DA) crosses.

analysis of this backcross should only reveal dominant and protective QTLs coming from the E3 strain. However, it has been observed that the progeny in genetic crosses may present more extreme phenotypes than observed in either parental strain, thus making it possible to also identify arthritis-promoting genes of E3 origin.

In the DA(E3 × DA) backcross, arthritis phenotypes were determined by the day of onset of disease, clinical score during the experimental period, maximal arthritis severity, and diameter measurements of the hind paws at the day of experimental termination. All linkage analyses were performed using the software Map Manager QTX (39) with the use of permutation tests (40) to determine individual threshold levels for each analyzed trait. As a reference for a Mendelian inheritance trait, we include the white-belly (WB) phenotype that was scored as an area of white fur on the belly and front wrists of ~50% of the rats. This phenotype was easy to determine and represents a phenotype with dominant inheritance. When using this phenotype as a quantitative trait, a single sharp linkage peak was obtained on chromosome 14 with an LOD score of 264 (Fig. 2). This simple Mendelian inheritance pattern should be compared with the more complex inheritance of the arthritis phenotypes that are more difficult to score, because they are regulated by several gene regions and thus result in fewer significant LOD scores with broader peaks.

#### QTL identification in the backcross analysis

Two loci reaching highly significant LOD scores for dominant protective E3-mediated effects on clinical severity coincided with previously reported PIA loci, namely *Pia7* (24) on chromosome 4 with an LOD score of 5.3 and *Pia4* on chromosome 12 with an LOD score of 53.1 (27). In addition, a dominant locus delaying arthritis onset and affecting early severity was identified on chromosome 20 with an LOD score of 4.8 (Table II, Fig. 3). Interestingly, this confirms *Pia1*, which includes the MHC region, and suggests that different haplotypes are associated with different phases of the disease, because MHC was previously associated



**FIGURE 2.** LOD score plots of the nominal phenotype of a patch of white fur on the belly of the otherwise dark-brown rats. This locus represents a monogenic trait. The thin vertical line shows the threshold for highly significant linkage (LOD of 4.4) as determined by permutation analysis (1000 permutations).

with chronic disease (17). Five previously unidentified QTLs that control arthritis severity were identified: *Pia13* on chromosome 7 with an LOD score of 3.3, *Pia14* on chromosome 8 with an LOD score of 3.4, *Pia10* on chromosome 10 with an LOD score of 3.1, and *Pia15* with a dominant effect on severity on chromosome 18 with an LOD score of 3.4 (Table II, Fig. 4).

As shown in Table II, the arthritis phenotypes correlate with each other. For example, clinical arthritis cosegregates with day of disease onset. However, the thickness of the hind paw ankles measured on day 28 is a combined estimation of edema caused by active inflammation and new cartilage and bone growth due to healing in the joint, whereas visual scoring mainly reflects active arthritis by edema and rubor. A new E3 dominant arthritis promoting linkage to chromosome 6 (*Pia12*), regulating swelling of ankles, was identified with an LOD score of 3.9 (Fig. 5). We have previously reported a QTL on chromosome 6, denoted *Pia3* (27), that peaks at the marker D6Wox5. However, we also identified the *Pia3* QTL in an intercross between DA and the recombinant inbred strain DXEC. In DXEC, the middle region of chromosome 6 between D6Rat34 and D6Wox5 is DA homozygous and therefore uninformative in the analysis. Hence, we reached the conclusion that these QTLs are separate and denoted the new QTL *Pia12*, even though they, unlike other QTLs in the present study, are E3 dominant for the disease phenotypes. We could also identify *Pia7*

Table I. Clinical scoring of PIA in parental rat strains and segregating crosses<sup>a</sup>

Strain	n	Incidence (%)	Maximum Score <sup>b</sup>	Affected Paws <sup>c</sup>	Arthritis Onset <sup>d</sup>
DA	104	100	32 ± 0.7	3.3 ± 0.1	12 ± 0.2
E3	10	0	0	0	—
(E3 × DA) <sub>F1</sub>	82	59	9 ± 1.1	1.7 ± 0.2	23 ± 1.0
DA(E3 × DA)	650	70	14 ± 0.6	2.1 ± 0.1	16 ± 0.2

<sup>a</sup> Clinical arthritis of parental rat strains and segregating crosses; all quantifications are presented as means ± SEM.

<sup>b</sup> Maximal observed arthritis severity.

<sup>c</sup> Mean number of affected paws in each rat with clinical signs of arthritis at the time of most severe arthritis.

<sup>d</sup> Day of arthritis onset, including only rats that developed disease.

Table II. Linkage analysis of DA(*E3* × *DA*) *PIA*

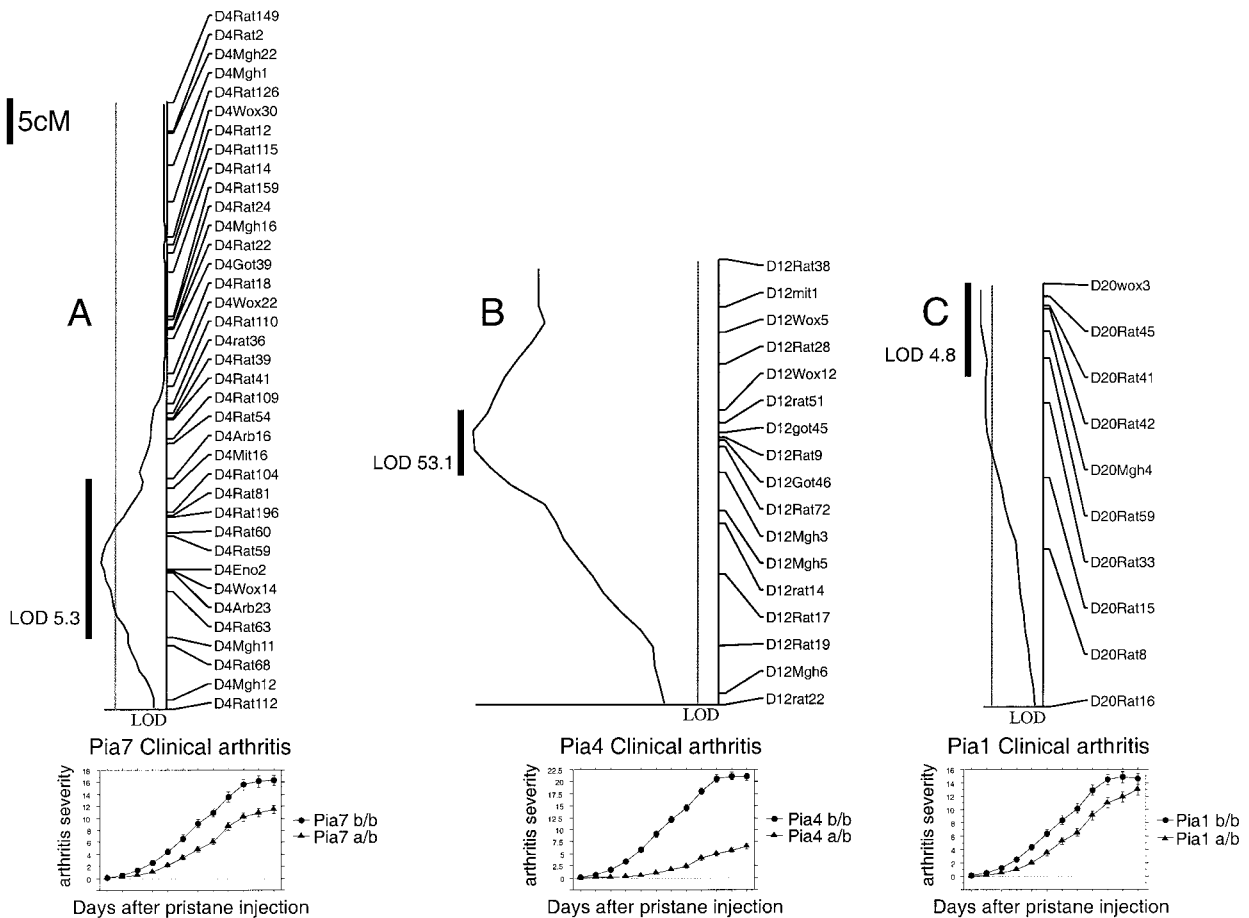
Phenotype	Marker	Inheritance	LOD	Significance	Level <sup>a</sup>	Locus	Related QTL <sup>b</sup>
Clinical score	D4arb23 <sup>c</sup>	DA-promoting	5.3			<i>Pia7</i> (24)	<i>Cia13</i> (19), <i>Oia2</i> (45)
	D7mit2	DA-promoting	3.3			<i>Pia13</i>	<i>Cia4</i> (26)
	D8mgh11 <sup>c</sup>	DA-promoting	4.0			<i>Pia14</i>	<i>Cia6</i> (46)
	D10mgh10 <sup>c</sup>	DA-promoting	3.1			<i>Pia10</i>	<i>Cia5</i> (26), <i>Oia3</i> (45)
	D12rat72 <sup>c</sup>	DA-promoting	53.1			<i>Pia4</i> (27)	<i>Cia12</i> (19)
	D18mit6 <sup>c</sup>	E3-promoting	3.4			<i>Pia15</i>	<i>Cia17</i> (46)
	D20wox3 <sup>c</sup>	DA-promoting	4.8	2.7	4.4	<i>Pia1</i> (17)	<i>Cia1</i> (26), <i>Oia1</i> (45), <i>Aia1</i> (22)
Onset	D4arb23	DA-promoting	3.0			<i>Pia7</i> (24)	<i>Cia13</i> , <i>Oia2</i>
	D8mgh11	DA-promoting	3.2			<i>Pia14</i>	<i>Cia6</i>
	D12rat72	DA-promoting	50.6			<i>Pia4</i> (27)	<i>Cia12</i>
	D20rat45	DA-promoting	3.3	2.9	4.3	<i>Pia1</i> (17)	<i>Oia1</i> , <i>Aia1</i>
Paw swelling	D4mit16	DA-promoting	3.2			<i>Pia7</i> (24)	<i>Cia13</i> , <i>Oia2</i>
	D6rat16 <sup>c</sup>	E3-promoting	3.9			<i>Pia12</i>	
	D7mit2 <sup>c</sup>	DA-promoting	4.7			<i>Pia13</i>	<i>Cia4</i>
	D12rat72	DA-promoting	32.0			<i>Pia4</i> (27)	<i>Cia12</i>
	D18mit6	E3-promoting	2.9	2.8	4.1	<i>Pia15</i>	<i>Cia17</i> (46)
COMP	D12Rat72 <sup>c</sup>	DA-promoting	27.0	2.8	4.1	<i>Pia4</i> (27)	<i>Cia12</i>
$\alpha_1$ -AGP	D12Rat72 <sup>c</sup>	DA-promoting	22.2	2.8	4.1	<i>Pia4</i> (27)	<i>Cia12</i>
WB <sup>d</sup>	D14Rat14 <sup>c</sup>	DA-promoting	264	2.9	4.4	WB, <i>Pia6</i> (27)	
CD4:CD8	D20wox3 <sup>c</sup>	DA-promoting	6.7	2.7	3.8	<i>Pia1</i> (17)	<i>Cia1</i> , <i>Oia1</i> , <i>Aia1</i>

<sup>a</sup> Significant and highly significant threshold levels for linkage determined by permutation test.

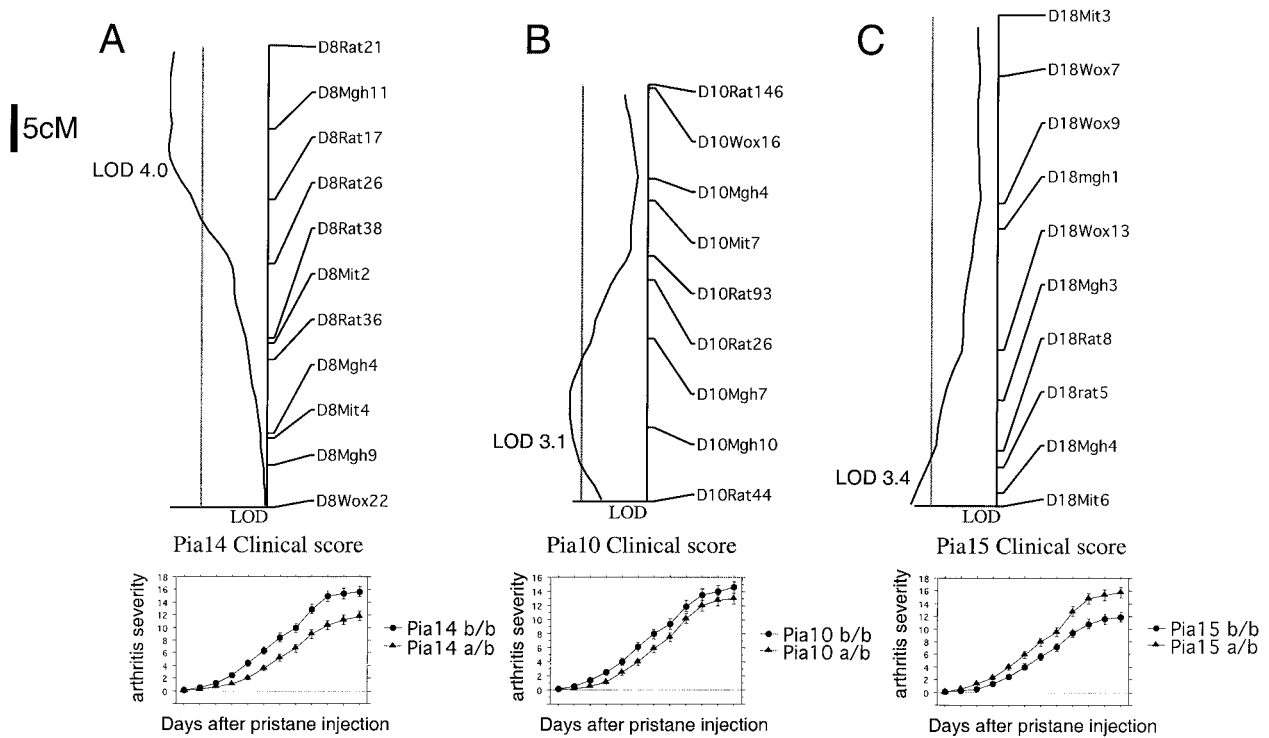
<sup>b</sup> Corresponding QTL in other rat models of arthritis.

<sup>c</sup> Shown in graph images.

<sup>d</sup> WB, White belly.



**FIGURE 3.** LOD score plots of *Pia7* (A), *Pia4* (B), and *Pia1* (C) for clinical arthritis scoring. The thin vertical line shows the threshold for highly significant linkage (LOD of 4.4), as determined by permutation analysis (1000 permutations). Short black vertical bars indicate the location of the previously reported assignment of corresponding *Pia* loci. The lower panel of each LOD plot reveals the disease phenotype in backcrossed rats stratified for genotype at *Pia7* (A), *Pia4* (B), and *Pia1* (C), respectively (a, *E3* allele; b, *DA* allele).

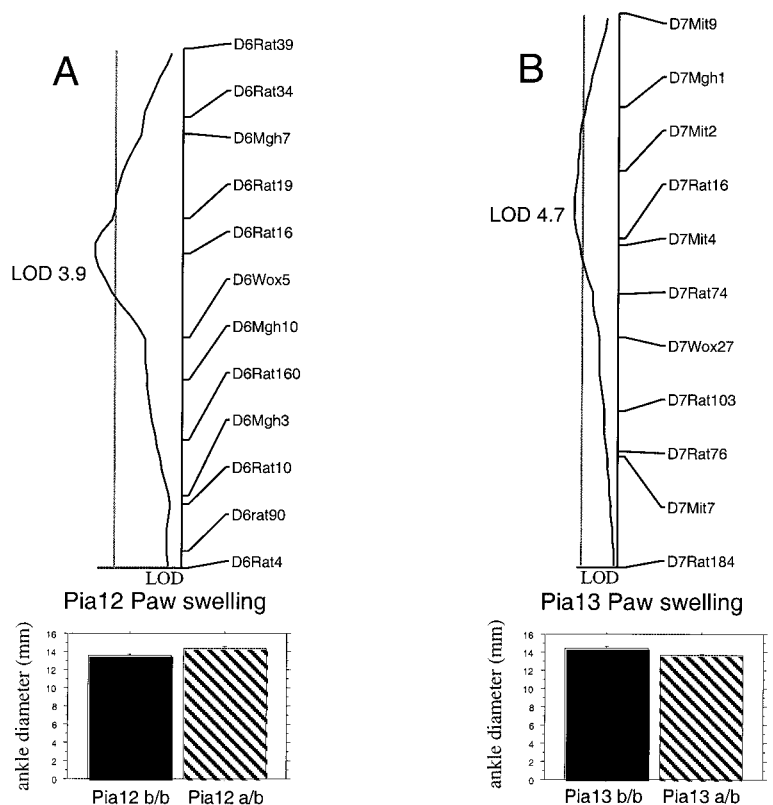


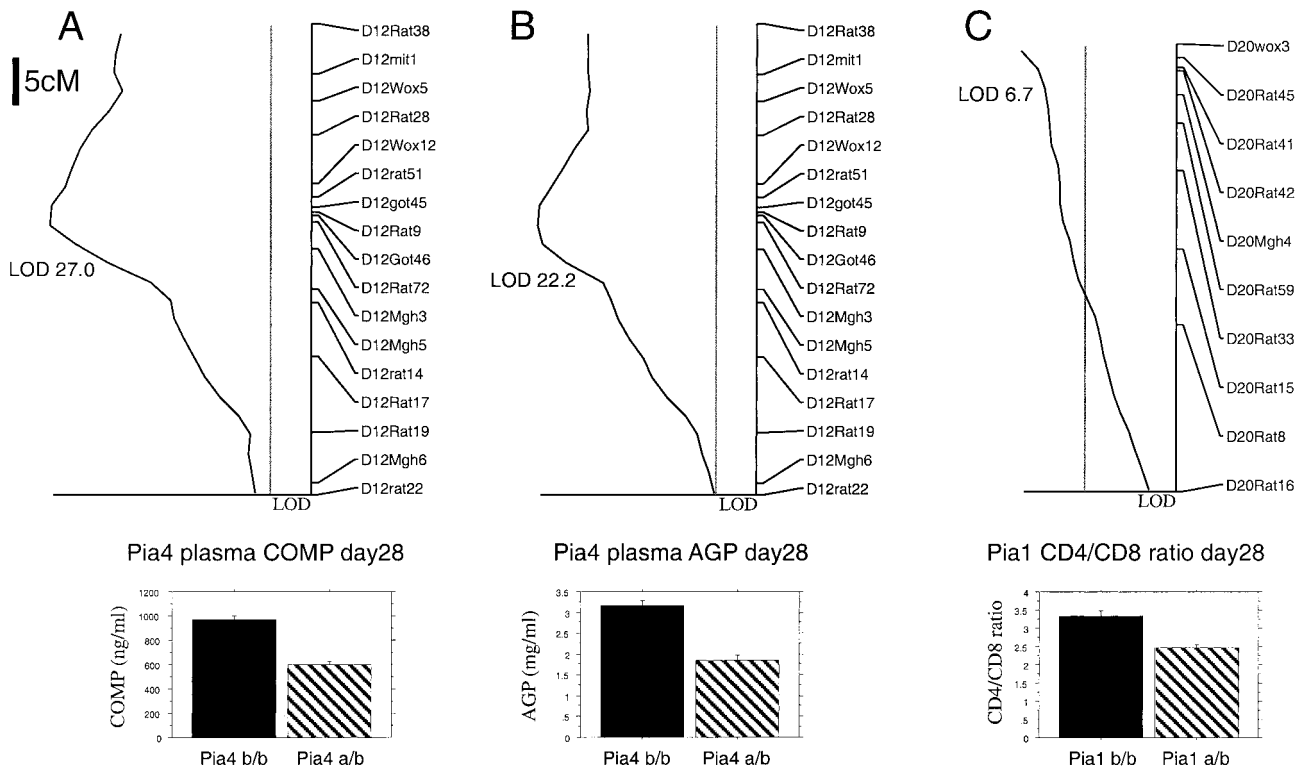
**FIGURE 4.** LOD score plots of *Pia14* (A), *Pia10* (B), and *Pia15* (C) for clinical arthritis scoring. The thin vertical line shows the threshold for significant linkage (LOD of 2.7) as determined by permutation analysis (1000 permutations). The lower panel of each LOD plot reveals the disease phenotype in backcrossed rats stratified for genotype at *Pia14* (A), *Pia10* (B), and *Pia15* (C), respectively (a, *E3* allele; b, *DA* allele).

(chromosome 4) and *Pia15* (chromosome 18) as significantly linked, and *Pia4* (chromosome 12) and *Pia13* (chromosome 7) as highly significant linked to the phenotype of paw swelling (Table II).

In addition to clinical arthritis phenotypes, we continuously investigate subphenotypes that are relevant for arthritis. In this experiment, we measured the plasma levels of COMP, previously associated with *Pia4* (chromosome 12) in acute disease and *Pia6*

**FIGURE 5.** LOD score plots of *Pia12* (A) and *Pia13* (B) for the sum of the diameter of the hind paws measured on day 28. The thin vertical line shows the threshold for highly significant (LOD of 4.1) (*Pia12* significant (LOD of 2.8)) linkage as determined by permutation analysis (1000 permutations). The lower panel of each LOD plot reveals the disease phenotype in backcrossed rats stratified for genotype at *Pia12* (A) and *Pia13* (B), respectively (a, *E3* allele; b, *DA* allele).



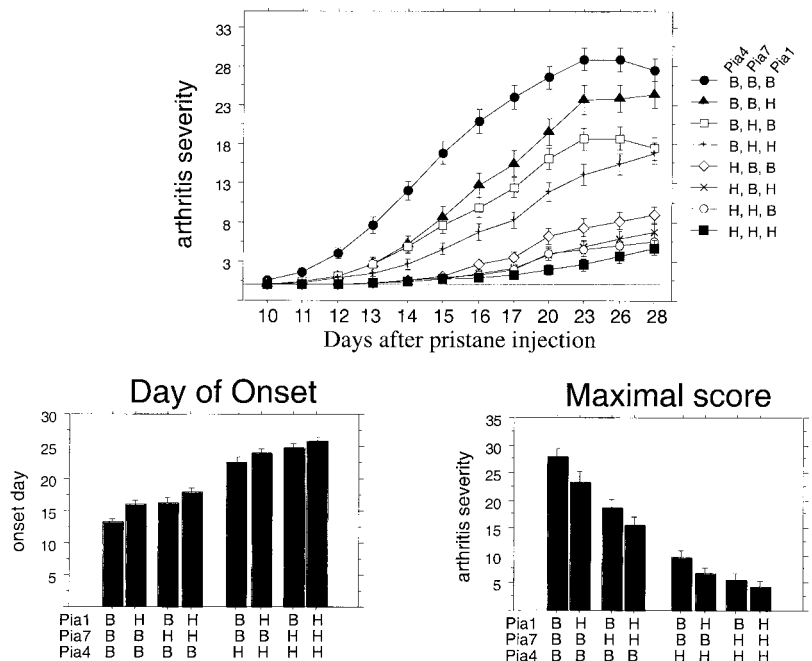


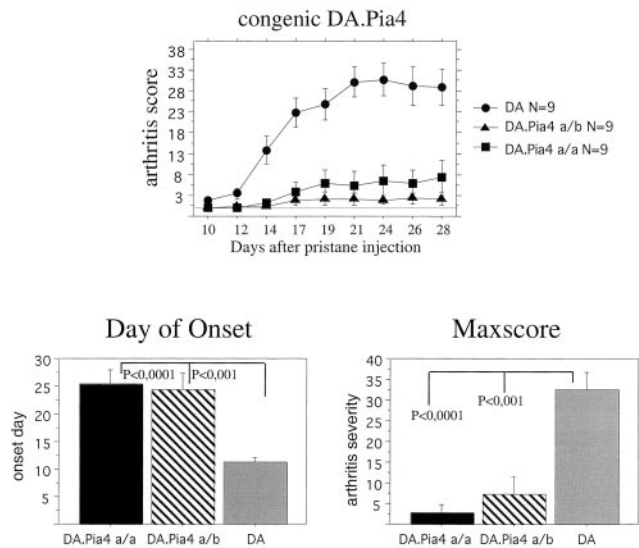
**FIGURE 6.** LOD score plots of *Pia4* with plasma levels of COMP (A), *Pia4* with plasma levels of AGP (B), and *Pia1* for CD4:CD8 ratio (C). The thin vertical line shows the threshold for highly significant linkage (LOD of 4.1) as determined by permutation analysis (1000 permutations). The lower panel of each LOD plot reveals the disease phenotype in backcrossed rats stratified for genotype at COMP (A) and AGP (B) with *Pia4* and CD4:CD8(C) ratio with *Pia1* (a, E3 allele; b, DA allele).

(chromosome 14) in chronic disease (27), and the plasma level of  $\alpha_1$ -AGP, previously linked to *Pia4* (chromosome 12) in acute disease (25). We also analyzed the ratio between CD4 and CD8 T cells in spleens in line with reports indicating CD4 T cells are the major T cell subset of importance for arthritis induction (41). With these subphenotypes, we believe we have identified three different aspects of the disease as the arthritis effector cells (CD4 T cells),

the acute systemic inflammatory response (AGP), and as the final outcome of the arthritis, peripheral joint destruction (COMP). Both the inflammatory response (AGP) and the cartilage destruction (COMP) were linked to *Pia4* (chromosome 12) with LOD scores of 22.2 and 27.0, respectively. The CD4/CD8 phenotype revealed linkage to the MHC region *Pia1* (chromosome 20) with an LOD score of 6.7 (Table II, Fig. 6).

**FIGURE 7.** Combined effect of *Pia4*, *Pia7*, and *Pia1* on clinical arthritis severity in the DA(E3  $\times$  DA) experiment, stratified for genotype at *Pia1*, *Pia7*, and *Pia4*, respectively (B, DA homozygous; H, heterozygous at the allele).





**FIGURE 8.** Verification of arthritis-regulating QTL in DA. *Pia4* congenic strain (a, *E3* allele; b, *DA* allele). Significance analysis of onset of disease and maximal arthritis severity was performed using the nonparametric Mann-Whitney *U* test.

*Isolation of QTLs in congenic strains*

The three major QTLs with strong effects on clinical arthritis (Fig. 7) as well as arthritis-related subphenotypes were the *Pia1* (chromosome 20), *Pia4* (chromosome 12), and *Pia7* (chromosome 4). These three QTLs were selected for congenic breeding with the DA strain as background and introgression of an arthritis-protective *E3* fragment containing *Pia1*, *Pia4*, and *Pia7*, respectively. The resulting congenic DA.Pia4 (D12Got46 to D12Rat26) rat (N14) showed, in accordance with the linkage analysis, 80% less severe arthritis with one allele of *Pia4* and 90% less severe arthritis with two alleles of *Pia4* (Fig. 8).

The *Pia4* locus in a DA background was as highly penetrant in the congenic *Pia4* strain as was observed in the DA(*E3* × DA) backcross (Table II). Therefore, a stratified approach on the available 650 DA(*E3* × DA) animals was used, in which all animals

heterozygous in the *Pia4* region were excluded in the material, leaving 322 animals for further analysis. In this analysis, no additional significant QTL was detected, but the significance of already detected loci was increased. *Pia1* and *Pia7* now increased to highly significant linkages with LOD of 6.5 and 8.6, respectively, using the phenotype of clinical arthritis. This argued for stronger effects of these two loci in the DA background, without the influence from *Pia4*. The DA.Pia7 (D4Mit16 to D4Mgh11) congenic strain carrying the *E3* allele of *Pia7* showed, after six generations of backcrossing (N7), strong protection against PIA. One *E3* allele in *Pia7* in the DA background was sufficient to reduce the level of arthritis severity by ~50% (Fig. 9).

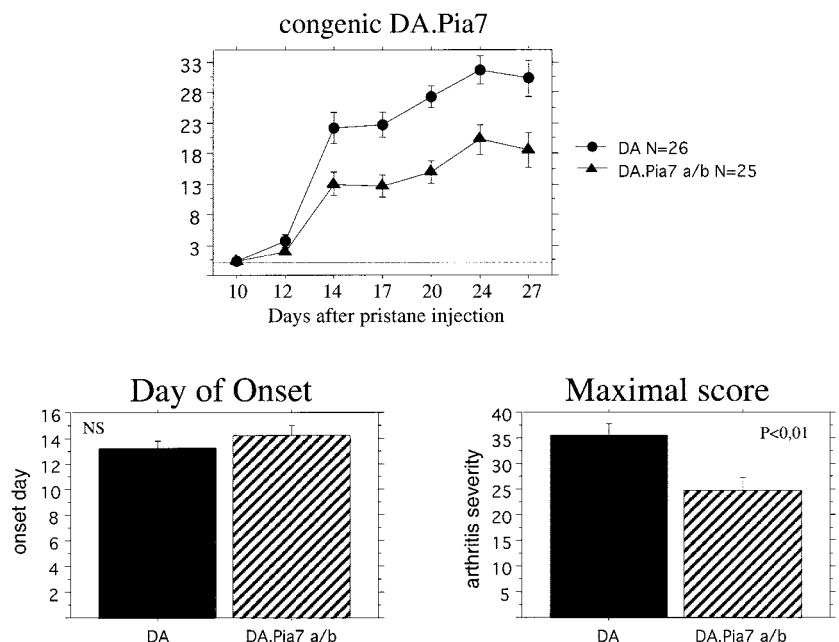
In the case of *Pia1*, a slightly different approach was used by measuring the subphenotype CD4:CD8 ratio, linked to the induction of disease. This ratio showed stronger correlation to disease than the clinical arthritis trait. The DA.Pia1 congenic rat was established through speed congenic techniques (33). In the case of DA.Pia1 (D20Wox3 to D20Mhg4), the background genome was determined to be pure after six generations of backcrossing to DA. In these animals, only the *Pia1* region including the MHC region was of *E3* origin. Before the induction of PIA in these congenic rats, blood samples were analyzed in FACS for the CD4:CD8 ratio. This analysis showed a highly significant ( $p < 0.0001$ ) additive effect on the CD4:CD8 ratio with the DA.Pia1 a/a congenic strain having the lowest cell ratio in peripheral blood. We also confirmed the difference in early disease severity, in which one or two alleles resulted in a decrease of arthritis severity by 20% ( $p < 0.05$ ) (Fig. 10).

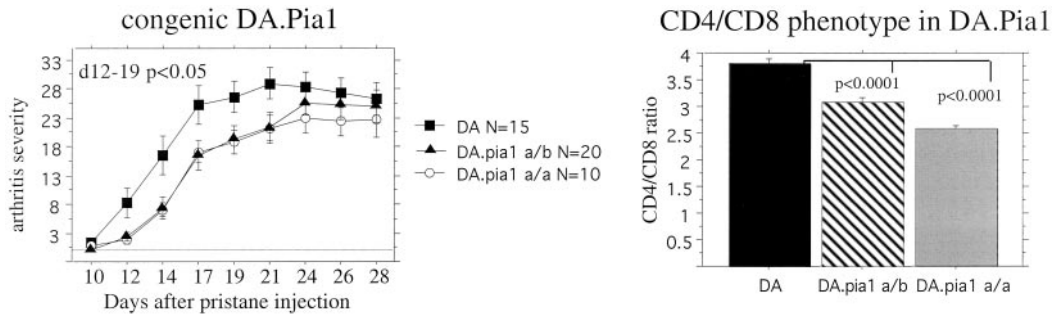
**Discussion**

In this backcross analysis, we observed that every second rat had an area of white fur covering its abdomen and front wrists. This trait was used in the linkage analysis as an example of fully penetrant Mendelian inheritance. In a QTL analysis, it is of interest to use the inheritance of a completely penetrant trait such as the white belly to compare with disease phenotypes that have a more complex inheritance, such as arthritis.

To break up the genetic complexity of arthritis into simple Mendelian traits, we use the PIA model (17) in rats. The inheritance of

**FIGURE 9.** Verification of arthritis-regulating QTL in DA.Pia7 congenic strain (a, *E3* allele; b, *DA* allele). Significance analysis of onset of disease and maximal arthritis severity was performed using non-parametric Mann-Whitney *U* test.



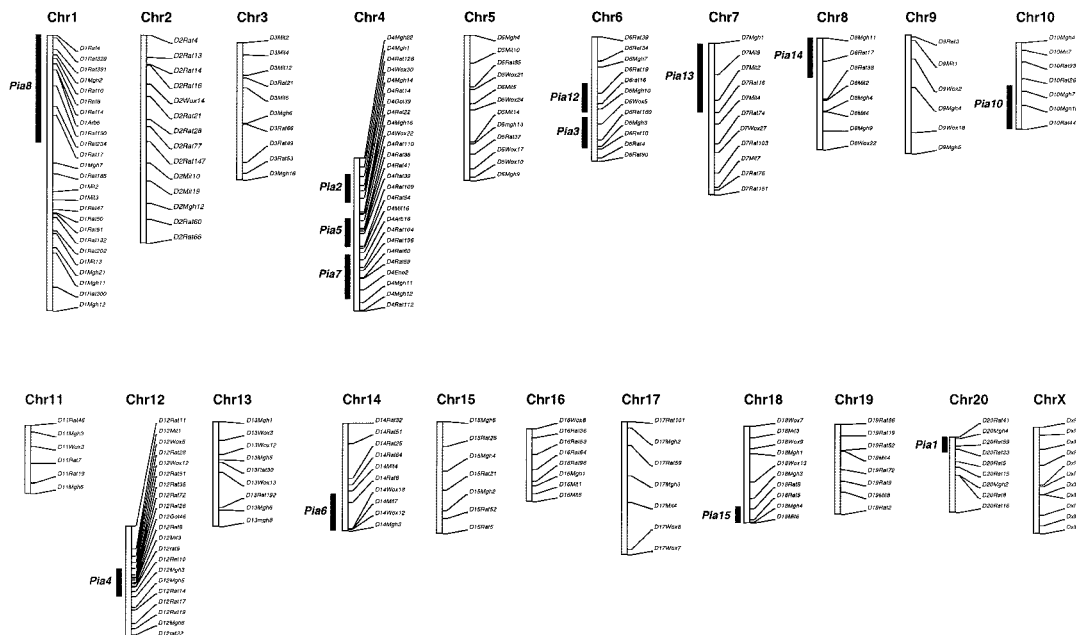


**FIGURE 10.** Verification of arthritis-regulating QTL in DA.Pia1 congenic strain (a, *E3* allele; b, *DA* allele). Significance analysis of CD4:CD8 ratio in blood leukocytes was performed using nonparametric Mann-Whitney *U* test.

PIA in our experimental system is complex, although it is determined by genomes from two inbred strains, i.e., the arthritis-resistant *E3* and the 100% susceptible *DA* rat strains. As a clinical score for PIA, we use severity of the disease as well as biochemical markers indicating systemic inflammation ( $\alpha_1$ -AGP) and cartilage erosion (COMP) as parameters for evaluation of PIA. The approach we have used since the first reported linkage analysis in this model (27) is to categorize the different phases of PIA as onset, early severe arthritis, and chronic continuous disease. QTLs regulating different aspects of PIA are then isolated in congenic strains, to eventually positionally clone the gene.

In this report, we describe a strategy involving (*E3* × *DA*)*F*<sub>1</sub> rats backcrossed to *DA* rats that increases the power of detecting *E3* dominant genes, compared with a conventional *F*<sub>2</sub> intercross. Also, by using as many as 650 progeny, we increased the QTL detection power and made possible the detection of additional significant QTLs (*Pia12* to *Pia15*; Table II) not previously identified in PIA, as well as previously identified *Pia* loci (*Pia1*, *Pia4*, and *Pia7*) (Fig. 11). The experimental design aimed at identifying strong dominant loci operating early in the disease. Therefore, as the experiment was terminated (day 28) shortly after maximal arthritis was reached (approximately day 24), loci associated with chronic disease, i.e., *Pia5* and *Pia6*, could not be detected. The animals were examined frequently during the onset period to as-

sess QTLs with a specific association to triggers for disease, i.e., *Pia2* and *Pia3*, previously recognized as onset-specific QTLs (27). Surprisingly, we were able to detect neither *Pia2* nor *Pia3* or to identify any QTL specifically regulating onset. We believe that this is due to the difference between the *F*<sub>2</sub> intercross and an *F*<sub>2</sub> backcross. This difference can be explained by a broader spectrum of disease onset in the intercross data, in which more genetic interactions are possible. In the backcross experiment, the strongest associations were obtained for genetic regions controlling early arthritis severity. The strongest linkages were obtained for *Pia1*, *Pia4*, and *Pia7*. Of interest for these three previously identified QTLs (17, 24, 27) is that *Pia1* has previously been assigned as a regulator of chronic diseases in MHC congenics with LEW background. This suggests the possibility of more than one gene in the *Pia1*/*MHC* region with impact on different aspects of the disease progression. Also of particular interest in this backcross experiment are the new *Pia12* and *Pia15* loci, because they present gene regions with *E3* alleles contributing to a more severe disease. In chromosome 6, where *Pia12* was identified, we already have identified a locus (*Pia3*) (27) with an *E3* dominant inheritance on the day of onset of PIA. However, in that report, we also presented suggestive association with the region corresponding to *Pia12* with an *E3* dominant effect on the number of affected paws with arthritis. Together with the fact that *Pia3* was also discovered in a cross



**FIGURE 11.** PIA linkages in *E3* × *DA* crosses subjected to PIA.

with the recombinant inbred strain DXEC, in which the *Pia12* region is excluded, this makes the annotation of two E3 dominant inherited QTLs in chromosome 6 (*Pia3* and *Pia12*) strong.

The three strong PIA QTLs (*Pia1*, *Pia4*, and *Pia7*) were selected for isolation and characterization in congenic strains. The most powerful QTL in this backcross experiment was *Pia4* (Table II), which was associated with all clinical arthritis phenotypes as well as the subphenotypes COMP (cartilage destruction) and  $\alpha_1$ -AGP (systemic inflammation). The introduction of the *E3* allele into the susceptible DA genome also resulted in an amelioration of arthritis severity by as much as 90%. Positional cloning and functional proof of *Ncf1* as the polymorphic gene in the *Pia4* QTL has recently been published (32). *Ncf1* is an important part of the NADPH oxidase complex of leukocytes responsible for free radical production in response to signaling events induced by invading pathogens and other immunological challenges (43). The polymorphic difference of *Ncf1* between E3 and DA rats results in a reduced function of the NADPH oxidase complex in the susceptible DA strain. This finding has several important implications. First, it confirms the use of linkage analysis and congenic strains to identify arthritis-regulating genes. Second, it identified a gene with an unanticipated effect in arthritis, especially because the identified effect pointed out the existence of the negative effects of low levels of free radical production in secondary lymphoid organs. As the impact of *Pia4* was so dramatic in the linkage analysis of the backcross experiment between E3 and DA rats, we used the power of having a large cohort of animals by stratifying the material to exclude animals affected by the *Pia4* QTL, thus leaving one-half of the animals for further linkage analysis. The result of this maneuver was an increase in significance for other QTLs, especially *Pia1* and *Pia7*, whose LOD scores increased to 6.5 and 8.6, respectively. Both the *Pia1* and the *Pia7* were reproduced in congenic lines and were shown to reduce arthritis severity by ~20% or 50%, respectively. Together with the beneficial effect of *Pia4*, these loci constitute the major part of susceptibility to PIA in DA rats (Fig. 7). One can envision that a combined triple congenic strain containing all of these loci (i.e., DA.Pia1, -4, and -7) would be totally resistant to PIA. This approach of combining QTLs into double or triple congenic strains has been used with success in other models of arthritis (44) and autoimmunity (45). Therefore, it is an appealing thought that only three polymorphic genes would be the key answer to arthritis susceptibility in DA rats in a polygenic arthritis model like PIA. However, one must bear in mind that this linkage analysis and the following characterization of the identified QTLs were performed only with the DA genome as the basis for the genetic evaluation. By doing the linkage analysis in a backcross to DA, only dominant genes from the E3 rats that interfere with the arthritis susceptibility of DA rats were discovered. What is missing is the reciprocal analysis with an identification of the arthritis-protective alleles with recessive inheritance of the E3 genome. Previous F<sub>2</sub> linkage analysis of the E3 and DA rat in PIA (24, 27) did not identify such genes. However, besides the DA.Pia congenic lines, we have also produced lines of E3.Pia4 and E3.Pia7 congenic rats as well as double E3.Pia4+7 congenic rats. Although *Pia4* and *Pia7* were, in this and other studies, identified to be the major susceptibility loci of PIA in DA rats, homozygous introgression of these gene regions into the resistant E3 rat failed to induce susceptibility to PIA (data not shown). This phenomenon indicates the complexity of polygenic diseases, even in the simplified genetics of only two genomes.

The association of genetic regions, or QTLs, with important features of complex diseases such as arthritis and the ensuing identification of disease-regulating genes through reverse genetics will provide the necessary knowledge in the effort to understand the

etiology of these kinds of disorders. Attempts to identify individual genes of complex diseases in humans are extremely difficult. Therefore, the use of adequate animal models of the studied disease is necessary not only to identify the important genes but also to understand the pathogenic mechanism leading to disease.

There exist several rodent models of RA, in which several QTLs have been identified and reproduced in congenic strains. Even if different models of arthritis are used in addition to different strains of animals, the accumulated knowledge from linkage analysis about the inherited components and the mechanism studied in congenic strains will help us to understand the pathogenesis of arthritis. Taken together, all of the knowledge of arthritis gained from animal investigations will strongly help in understanding the inheritance of RA in humans. Another importance of animal models is the possibility, not available by other means, to further study the effects of the identified arthritis-regulating genes in actual disease and thereby facilitate the development of target-specific drugs based on knowledge of the disease-inducing parameters in an in vivo situation.

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