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Both common and unique susceptibility genes in different rat strains with pristane-induced arthritis

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Pristane-induced arthritis (PIA) in rats is an animal model for rheumatoid arthritis (RA). We have previously identified seven quantitative trait loci (QTLs), which regulate arthritis development using a cross between the susceptible DA strain and the resistant E3 strain of rats (*Pia2–8*). In the present study the inbred rat strain LEW.1F was used as the susceptible strain in a cross with the E3 strain. The results confirmed the locus *Pia4* on chromosome 12, which previously was shown to be associated with PIA, and also with experimental allergic encephalomyelitis, in crosses between the rat strains E3 and DA. On chromosome 1, linked to the albino locus, we identified a novel QTL, *Pia9* in the LEW.F1 cross. This locus was associated with arthritis severity in the early phase of disease. A locus on chromosome 16, denoted *Pia11*, was also associated with arthritis severity in the early phase of the disease. A suggestive locus was detected on chromosome 14, which was associated with arthritis severity at the time when PIA progresses into a chronic phase. Using a congenic LEW.1F strain, which carries E3 alleles at the *Pia9* locus, we confirmed that the E3 allele significantly suppresses arthritis severity during the early phase of the disease. The results revealed synergistic effects between different susceptibility loci using ANOVA analysis. These interactions were influenced by gender. Rats with *Pia9* alleles from LEW.1F and *Pia11* alleles from E3, were shown to suffer from much more severe arthritis in the early stage of the disease. On the other hand, the *Pia9* and the suggestive locus on chromosome 14 affected only males during the chronic phase of the disease. These findings provide clues to how genetic factors by themselves, and in interaction with each other, regulate the development of a disease, which displays many similarities to RA.

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Introduction

Rheumatoid arthritis (RA) is considered to be an autoimmune disease affecting about 1% of the population worldwide.¹ The etiology of this complex disorder is unknown, and the genetic contribution is obscured by strong environmental factors.² The most intensively studied genetic factor associated with RA is the major histocompat-

ibility complex (MHC, or HLA in humans).^{3–7} The MHC accounts for 30–60% of the variability, based on λ_{MHC} estimates in various populations.^{8–11} RA is considered to be an autoimmune. The genetic regulation of autoimmune diseases is poorly understood and at least three models have been proposed, a multiplicative model,¹² a polygenic threshold liability model¹³ and a heterogeneity model.¹⁴

Recently, two whole-genome scans, involving a wide range of RA families in Europe and US, identified linkage to several chromosomal regions, including the MHC class II region. However, only the linkage to the MHC-region reached significance at the genome wide level.^{15,16} The

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completion of the human genome sequence gives an impetus to identify genes involved in complex diseases like RA. RA is, however, a heterogeneous disease with low penetrance, influenced by different environmental factors. Moreover, the prevalence in different ethnic populations varies. This makes it exceedingly difficult to clone the predisposing genes based on genetic studies of RA patients. An alternative is to use animal models, which allow the environmental factors as well as the genetic background to be controlled.

Several animal models for RA are well established in mice and rats, including mycobacteria-induced arthritis (MIA), collagen-induced arthritis (CIA), oil-induced arthritis (OIA), pristane-induced arthritis (PIA), and proteoglycan-induced arthritis (PgIA).¹⁷ These models mimic the human disease in many respects. Genetic studies of the models have demonstrated that MHC II genes play a crucial role in the development of the disease, as is the case for RA. An important advantage is that the animal models can be used to identify predisposing genes outside of the MHC locus. A large number of susceptibility loci have been identified which regulate arthritis development based on studies of CIA and PgIA in mice, and CIA, PIA, MIA and OIA in rats.^{13,18–22} These findings infer that the genetic regulation of arthritis is much more complicated than previously anticipated. There are, for instance, at least nine loci that control time of onset, severity, and chronicity of PIA in rats.^{18,23} Another important finding was that some loci seem to be shared among animal models for different autoimmune disorders, and also shared among different species, like mice, rats, and even humans.^{18,24–28} Finally, the effect of the QTLs depends on strain, gender, and different induction protocols and other environmental factors.

To limit the complexity we have selected the PIA model, to study the segregation of genetic factors in crosses between different susceptible and resistant animal strains. PIA is induced by pristane, a non-immunogenic adjuvant, and resembles RA in many aspects.²⁹ Susceptible rat strains were injected with pristane intradermally, and the animals subsequently developed arthritis about 2 weeks after injection. The pathological changes included inflammatory cell infiltration, pannus formation, erosion and formation of bone and cartilage similar to what is seen in the joints of RA patients. It is known that $\alpha\beta$ T cells play a crucial role in the development of the disease, based on studies showing that antibody ablation can block the disease. Another important finding is that PIA, like RA, is associated with MHC.²⁹ It is noteworthy that the association to MHC was only observed in the chronic phase of the disease. MHC is, however, not the only locus controlling the disease, shown by the fact that the arthritis incidence varies among strains with identical MHC genes on different genetic backgrounds. Two crosses involving DA and E3 and DA and the recombinant inbred strain DXEC, respectively,^{18,23} revealed seven QTLs. It is intriguing that among these loci, some

control the arthritis onset (*Pia2* and *Pia3*), some regulate arthritis severity (*Pia4*, *Pia7* and *Pia8*), whereas others regulate chronicity (*Pia5* and *Pia6*). Although the crosses DA $\times$ DXEC and E3 $\times$ DA involve animals with very similar phenotypes, *Pia7* and *Pia8* were only revealed in the former cross, indicating complex interactions between the predisposing genes. Another locus on chromosome 10, denoted *Pia10*, was identified in a strain congenic for the *Cia4* locus (Ron Wilder, personal communication).

In this study, we used a cross between the susceptible strain LEW.1F and the resistant strain E3 to produce 181 F2 progeny. Arthritis was induced with pristane and four QTLs were found to regulate susceptibility to arthritis. Three loci, which have not been identified previously, were identified on chromosomes 1, 14 and 16, denoted *Pia9*, *Piax* and *Pia11*, respectively. The fourth locus, *Pia4*, on rat chromosome 12 has already been shown to be an important susceptibility locus for PIA in the E3 $\times$ DA cross, and displayed the same pattern of inheritance as seen previously.

Materials and methods

Rats

E3 and LEW.1F rats originating from the Zentralinstitut für Versuchstierzucht, Hannover, Germany, were kept and bred in a climate-controlled environment with 12 h light/dark cycles in our animal department under pathogen-free conditions. The rats were housed in polystyrene cages, each with two or three rats, containing wood shavings and fed standard rodent chow and water *ad libitum*. The E3 and LEW.1F rats were intercrossed to obtain (E3 $\times$ LEW.1F) F1 and F2 progeny. All experiments were performed with rats aged from 8 to 12 weeks. The rats were checked for the common pathogens including Sendai virus, Hantavirus, coronavirus, reovirus, cytomegalovirus and mycoplasma pulmonis.

Induction and evaluation of arthritis

Pristane (2, 6, 10, 14-tetramethylpentadecane) (Aldrich, Milwaukee, WI, USA), 150 μ l per rat, was injected intradermally at the base of the tail under ether anaesthesia. Arthritis development was monitored using a macroscopic scoring system for each of the four limbs ranging from 0 to 4 as follows: 1=swelling and/or redness in one joint; 2=two joints involved; 3=more than two joints involved; and 4=severe arthritis in the entire paw. The maximum total score is thus 16 for each rat by adding up the scores from four paws. The rats were observed 1–3 times a week during the experimental period.

Genotyping

DNA was prepared according to a standard protocol. Primer sequences for rat microsatellite markers defined as D $\times$ Mity, D $\times$ Raty and D $\times$ Goty were from Research Genetics (Huntsville, AL, USA), and for markers defined as D $\times$ Woxy from the Wellcome Institute for Human Genetics (Oxford, UK).

Fluorescent-labelled primers were synthesised by Interactiva Biotechnologie (Ulm, Germany). All the markers were assayed by PCR on ABI 877 Integrated thermal cycler (Perkin-Elmer) according to standard protocol; and PCR products were separated on an ABI 377 DNA Sequencer (Perkin-Elmer) and data analysed with software package GeneScan[®]3.1 and Genotyper[®]2.1 (Perkin-Elmer). For making the congenic strain, genotyping was performed using MegaBACE 1000 DNA Analysis System (Molecular Dynamics, USA) and data were analysed with software Genetic Profiler Version 1.1 (Molecular Dynamics, USA)

Arthritis induction in the *Pia9* congenic strain

To make the strain congenic for *Pia9*, the conventional strategy was used to introgress the resistant region from E3 rats onto the susceptible background of LEW.1F. During the first four generations, we selected black and white rats to backcross with LEW.1F, because the c-locus was found to co-segregate with the disease. Then a panel of microsatellite markers around the D1Rat50 was used to further select rats for backcrossing. The experiment was performed on N8 rats that contained an E3 fragment between c-locus and D1Mgh21 (<20 cM). The rats were injected with 100 μ l pristane intradermally in the base of the tail. Arthritis development was monitored 1–3 times until day 49 after pristane injection with the new macroscopic scoring system for the four limbs ranging from 0 to 15 points for each paw (1 point for each swollen or red toe, 1 point for mid-foot digit and knuckle, 5 points for a swollen ankle or wrist joint). In this way the scores of the four paws yields a maximum total score of 60 for each rat.

Statistical analysis

Linkage analysis was performed with the Mapmaker computer package.³⁰ A logarithm of odds (LOD) 3.9 was considered to be significant and above 2.8 as suggestive linkage, which is based on the guidelines suggested by Lander and Kruglyak.³¹ And we also used Map Manager QTXb13 software to perform permutation tests (<http://mapmgr.roswellpark.org/mmQTX.html>). In order to compare incidences between males and females, the Chi-

square test was performed. To compare the arthritis severity, indicated as clinical scores at the different time point after pristane injection, non-parametric analyses, Mann–Whitney test for two groups, and Kruskal–Wallis test for three groups or more, were used. Two-way ANOVA was used to test for interactions between two loci on the arthritis development. In all the analyses we used $\alpha=0.05$. The analyses were performed with the statistic software – StatView version 5.0.1 (SAS Institute, USA).

Results

Arthritis development in parental, F1, and F2 rats

The susceptibility to PIA of parental strains and the progeny from different crosses is presented in Table 1 and Figure 1. The LEW.1F rats were susceptible to PIA with an incidence of 100% in females, whereas E3 rats were completely resistant to PIA. The F1 rats were susceptible to PIA, but with a delayed onset, reduced severity and incidence. In the F1 population, only females developed disease, confirming the female preponderance in the LEW.1F strain. The F2 population exhibited a large variation in disease development after pristane injection. The severity of the disease was only slightly greater in females than in males.

Identification of arthritis QTLs

Four QTLs were identified in this cross and the results are shown in Table 2 and Figure 2. The detailed linkage map about microsatellite markers can be found in our homepage, <http://net.inflam.lu.se>. *Pia4* on chromosome 12, discovered previously in an (E3 $\times$ DA)F2 intercross, was identified also in this cross, confirming the presence of a protective allele in E3.¹⁸ In LEW.1F, as in DA, this locus regulated the clinical severity in both males and females (Figure 3C and D). Novel QTLs showing significant linkage with arthritis severity were found on chromosomes 1 (*Pia9*), and 16 (*Pia11*). In addition a locus on chromosome 4 (*Piax*) showed suggestive linkage (Figure 2). The predisposing allele at the *Pia9* locus originated from the LEW.1F strain, whereas at the *Pia11* the predisposing allele was derived from the resistant E3 strain (Figure 3). The locus on chromosome 14 (*Piax*), displayed overdominance, ie the

Table 1 Arthritis development in parental strains, F1 and F2 rats

	Sex	No	Incidence (%)	Onset day ^a	Max score ^a
LEW.1F	Male	14	86	31 $\pm$ 7	9 $\pm$ 1
	Female	8	100	18 $\pm$ 1 ^c	8 $\pm$ 1
E3	Male	5	0	–	–
	Female	5	0	–	–
(E3 $\times$ LEW.1F) F1	Male	4	0	–	–
	Female	5	80 ^b	38 $\pm$ 10 ^c	5 $\pm$ 2 ^c
(E3 $\times$ LEW.1F) F2	Male	94	52	72 $\pm$ 4	3 $\pm$ 0.5
	Female	87	56	69 $\pm$ 5	3 $\pm$ 0.4

^aIncluding all rats and expressing mean $\pm$ SEM; ^b $P < 0.05$ between male and female rats using Fisher's Exact Chi Square Test; ^c $P < 0.05$ between male and female rats using nonparametric Mann–Whitney Test.

Figure 1 Clinical scores (mean $\pm$ SEM) in the LEW.1F (A) ($E3 \times LEW.1F$) F1 (B) and F2 rats (C) after pristane injection (150 μ l/rat). Arthritis was monitored twice or thrice a week using a macroscopic scoring system for each of the four limbs ranging from 0 to 4. Shaded area represents a significant difference between females and males (Mann–Whitney test, $P < 0.05$).

heterozygotes had a more severe phenotype than either of the homozygotes (Figure 3). Gender differences were observed for the penetrance of the different QTLs; *Pia9* and *Piax* had a more pronounced effect in females and *Pia11* in males (Figure 3). It is shown that *Pia9* regulates the severity in early stage of the disease while others control disease severity in fully flaring phase (Figure 4). The loci should obviously be confirmed in congenic strains, which is a time consuming process. So far we have tested the *Pia9* locus after backcrossing of the protective E3 allele into LEW.1F for eight generations. The arthritis phenotype could be produced in the congenic strain, manifesting significantly less severe in the congenic animals (24 rats in total); but the data could not exclude the possibility that the *Pia9* locus in fact contains more than one gene associated with arthritis severity (Figure 5).

Interactions between the observed QTLs

We used two-way ANOVA to study interactions between the loci, choosing the most significant markers representing each locus after single marker analyses (Kruskal–Wallis test). The combined effects of pairs of loci on the clinical scores during different phases of the disease were measured. We found that the combination of *Pia9* and *Pia11* showed a synergistic effect. This effect was observed in both females and males during the acute phase of the disease (Figure 6A and B). Animals homozygous for the LEW.1F allele at the *Pia9* locus, and homozygous for the E3 allele at *Pia11* showed a more severe disease at day 18 after pristane injection than individuals homozygous at only one of these loci (Figure 6C). The combination between *Pia9* and *Piax*, showed a more complex pattern. Firstly, the combined effect could only be detected in male rats (Figure 7A and B). Secondly, male rats heterozygous at *Pia9* or *Piax*, suffered from much more severe arthritis (Figure 7C) later in the disease course in contrast to the females (Figure 7D).

Discussion

PIA is controlled by several genes as previously shown by segregation analysis of the ($E3 \times DA$) and ($DA \times DXEC$)

Table 2 Linkage of arthritic traits in chromosome regions with inherit modes in female and male rats from ($E3 \times LEW.1F$) intercross

QTLs	Chromosome	Affected Phenotype ^b	Marker ^c	all	Lod score		Inheritance pattern ^d	Variance explained (%)	Cosegregated phenotypes ^e
					females	males			
<i>Pia9</i>	1	Score day28	D1Mit2	5.3	3.7	2.2	LEW recessive	13	Max score (4.2) Total score (4.5)
<i>Pia4</i>	12	Score day40	D12Rat72	8.9	4.2	5.9	LEW recessive	20	Max score (7.6) Total score (5.8) Onset day (6.4)
<i>Piax</i> ^a	14	Score day47	D14Rat64	3.3	2.0	<2.0	$h > b > a$	8	–
<i>Pia11</i>	16	Score day35	D16Rat53	4.4	2.8	3.4	E3 recessive	19	Max score (3.1) Total score (3.0)

^abeing called *Piax* since Lod score is less than 3.9; ^bLod score of clinical score at different days after pristane injection is shown in Figure 4; ^cMarkers nearest QTLs; ^da stands for E3 allele, b for LEW.1F allele, h for heterozygous; ^eLod score values in parenthesis.

Figure 2 LOD score plots for chromosomes indicating identified OTLs. (A–D) represent LOD scores for Chromosome 1, 12, 14 and 16, respectively. The thresholds for suggestive, significant and highly significant linkages were determined after permutation test by using Map Manager QTXb13 software and indicated by dotted lines.

intercrosses.^{18,23} In this study, we used LEW.1F as the susceptible counterpart in a cross with the resistant strain E3, in order to study whether the same susceptibility loci were unraveled when different susceptible strains were used in the crosses. The *Pia4* locus which is on rat chromosome 12, was also identified in this cross and influenced the severity of the disease. The predisposing allele originated from the susceptible strain as was the case in the DA × E3 cross. Three novel QTLs were identified in the LEW.F1 cross which all influence arthritis development, were denoted *Pia9*, *Piax* and *Pia11*, and located on chromosomes 1, 14, and 16, respectively. The QTLs detected in the current study were all influenced by gender and were associated with arthritis severity. They interacted with each other and colocalise with QTLs of other autoimmune diseases.

Females are more susceptible to PIA, as also is the case for RA. Female rats of the susceptible parental strain, LEW.1F, have much higher clinical scores both during the acute and the chronic phases, but the incidence was almost the

same in the males. A female preponderance is in fact seen in both LEW.F1 and DA in other arthritis models such as avridine-induced arthritis and the type II collagen induced arthritis.³² However, this rule has exceptions since male rats are more susceptible to collagen type XI induced arthritis than female rats.³³ The observed gender difference appears thus to be disease specific and does not differ between the DA and LEW strains. The mechanisms behind sex association are likely to be complex, involving both sex chromosomes and sex hormones. Clearly, sex-specific effects on autosomal QTLs are commonly observed.^{34–37}

Previous studies have shown that the onset, severity, and chronicity of PIA are controlled by different sets of genes. In this study, we found that all loci were associated with severity although they tended to influence the phenotype at different time points. The onset of PIA in the LEW.1F is about 21 days after pristane injection, one week later than in the DA rats. Linkage to *Pia9* on chromosome 1 was detected on day 18 and reached the maximum LOD

Figure 3 Disease severity with different genotypes in females and males ($\bar{x} \pm \text{SEM}$). (A) stands for E3 homozygous, (B) for LEW.1F homozygous and (H) for heterozygous for linked markers (same as Figures 6 and 7). Shaded areas indicated the a significant difference among different genotypes (Kruskal–Wallis test, $P < 0.05$).

Figure 4 Lod score of 4 Pia OTLs with clinical score at different days after pristane injection. Suggestive and significant thresholds were 2.8 and 3.9 respectively suggested by Lander and Kruglyak.³¹

Figure 5 Confirmation of *Pia9* in congenic rats ($n=24$). The E3 fragment of *Pia9* has been backcrossed on the LEW.1F background and the *Pia9* heterozygous rats are compared with wild type LEW.1F rats for PIA development at day 28 after pristane injection at different markers along the congenic fragment. * $P < 0.05$ analysed with Mann–Whitney test.

on day 28. *Pia11* is also manifested early after pristane injection. Thus two loci seem to control the severity of the disease in the early phase. The suggestive locus, *Piax* on chromosome 14, seemed to act later in the disease. This was striking when it was analysed together with *Pia9*. *Piax* maps close to a previously identified locus associated with chronic arthritis, *Pia6*, but shows a different inheritance pattern. The observation that different genes are involved in the pathogenesis of PIA suggests that several different pathways are involved in disease development. It is also noteworthy that interactions between loci seem to be important for the arthritis development.

It is well known that the complex disease traits are polygenic. In this study, we utilised two-way ANOVA to analyse the pair-wise interactions between loci on the clinical scores during the course of the disease. Two loci combinations played crucial roles at the onset and during the chronic phase of the disease, respectively. One is the combination of *Pia9* and *Pia11*. This effect is seen in both female and male rats during the acute phase of the disease (Figure 6). Another interaction between *Pia9* and *Piax* regulated severity later in the disease course. Interestingly, when *Pia9* was combined with different QTLs it manifested different effects not only in different sexes but also during various phases of the disease. It will thus be necessary to construct double congenic animals to study the interactions, as has been done successfully in the SLE mouse

Figure 6 Interaction between *Pia9* and *Pia11*. (A) Clinical scores after pristane injection in the rats with different *Pia9* and *Pia11* genotypes in the female. Shaded area shows the significant difference ($P < 0.05$) analysed with Kruskal–Wallis test. (B) The same as A but in the male. (C) clinical scores at day 18 after pristane injection in all the rats with different combinations between *Pia9* (Marker D1Rat51) and *Pia11* (marker D16Wox8). Two-way ANOVA was used to analyse the significance and synergistic effects ($P < 0.01$).

Figure 7 Interaction between *Pia9* and *Piax*. (A) clinical scores after pristane injection in the rats with different *Pia9* and *Piax* genotypes in males. Shaded area shows the significant difference ($P < 0.05$) analysed with Kruskal–Wallis test. (B) The same as A but in females. Clinical scores at day 66 after pristane injection in males (C) and females (D) with different combinations between *Pia9* (marker D1Rat51) and *Piax* (marker D14Rat64). Two-way ANOVA was used to analyse the significance and synergistic effects showed in only males ($P < 0.01$) not females ($P > 0.05$).

model.³⁸ Another alternative is to first identify the candidate genes in the QTLs and then to study their relationship at the molecular level.

Pia9, on chromosome 1, colocalises with previously identified loci in the rat named *Cia2*, *Eae6*.^{22,26} In the homologous region on mouse chromosome 7, there is a locus associated with CIA, *Cia7*,¹⁹ and another associated with EAE, *Eae4*.³⁹ *Pia4* also colocalises with other QTLs for autoimmune disease, for example, *Eae5*, *Eau2* and *Cia12*.^{26,40,41} In a region on mouse chromosome 5, which is homologous to *Pia4*, association has been reported to arthritis severity of Lyme disease.⁴² These findings indicate that common susceptibility genes for several autoimmune diseases exist. However, there is no previously identified QTLs located in the regions of *Piax* and *Pia11*, suggesting that these may be specific for arthritis.

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