
BY

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ABSTRACT


Predisposition to autoimmune diseases such as, rheumatoid arthritis, diabetes, and multiple sclerosis, is caused by the effect of multiple genes and a strong influence from the environment.

In this study, I have investigated genetic factors that confer susceptibility to rheumatoid arthritis in a rat model. This work has led to the identification of several chromosomal regions, containing uncharacterized genes that directly or indirectly are associated to the arthritis development in these rats. We have observed that timing, gender, and genetic interactions are features that play a part in the effect that these genetic factors exert.

Unarguably, animal models for human disorders display differences to the human form of disease. An important fact is however that the same chromosomal regions are identified in both rodent and human studies, which suggests that there are genetic factors that we have in common, which are involved directly or indirectly with an autoimmune response.

Focusing the interest on these similarities, and on the possibility to apply a wide set of genetic tools, make animal models an invaluable, and probably necessary, instrument to dissect the genetic component of complex disorders. To fully comprehend the genetic basis for a complex disorder like this, will require understanding of how multiple genes interact with each other to cause disease.

We have been able to demonstrate that chronic arthritis, in a rat model for rheumatoid arthritis, is regulated by several genes and that these act during different temporal phases of the disease. These findings will hopefully contribute to our understanding of the etiology and progression of rheumatoid arthritis.

Key words: Complex disease, autoimmune, rheumatoid arthritis, genetic, mapping, QTL, locus, linkage, animal model, rat.

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List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


IV. Shemin Lu*, Niklas Nordquist*, Jens Holmberg, Peter Olofsson, Ulf Pettersson and Rikard Holmdahl. Identification of two novel non-MHC loci which control pristane induced arthritis in rats - confirmation in a congenic strain.


* These authors have contributed equally to the work.

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Abbreviations for animal models of autoimmune diseases

Aia Avridine induced arthritis
Bb *Borrelia Burgdorferi* induced arthritis
Cia Collagen induced arthritis
Eae Experimental allergic encephalomyelitis
Eau Experimental autoimmune uveitis
Idd Insulin dependent diabetes
Iddm Insulin dependent diabetes mellitus
Lmb Lupus in MRL x C57BL/6 mice
Lwb Lupus white and black
Oia Oil induced arthritis
Scwia *Streptococcus* cell wall induced arthritis
Sle Systemic lupus erythematosus
Introduction

Genetic disorders

Human genetic disorders occur as a consequence of abnormal functions in our genome. Which disorders then, are genetic disorders? In a broad sense, one could say that our genes to some extent regulate all diseases. Many times though, it is obvious that a disease is caused by a pathogen from the outside. It is for example a well-known fact that “the flu” is caused by the influenza virus. On the other hand, it is also well known that some people display mild symptoms, while others get more severely affected. Differences in the susceptibility to infectious agents can to some extent be ascribed to different individual genetic makeups (Dietrich, 2001).

In a more narrow sense, genetic disorders are commonly disorders that are caused by malfunctioning genes or gene-products, or disorders where genes contribute substantially to the risk of becoming affected. So, how does one determine whether genes are of importance for a particular disease?

Sibling relative risk

An indication of a genetic component involved in predisposition to a disease is an increased sibling relative risk. This is a measure of the risk that, given one affected sibling in a sibling pair, the second sibling is also affected. It should be noted though, that this measure also includes the effect of shared environment.

Twin studies

Most disorders have a genetic component that explains parts of the observed phenotypic variability. In some cases, a predisposition for a certain disease can be completely explained by genetic causes. Cystic fibrosis is a disease caused by one single defective gene, (Riordan et al., 1989). A carrier of the defective gene will, without exception, become affected. In other cases, the genetic component may explain only a small fraction of the total occurrence of a certain disorder. This can be exemplified with cervical cancer, where the genetic component has been estimated to account for 27% of the variation in liability to disease (Magnusson et al., 2000). One can assess the magnitude of the role that genetic factors play in a certain disorder by comparing concordance rates in monozygotic and dizygotic twins. Since monozygotic twins are genetically identical, and dizygotic twins share, on average, 50% of their genes, the difference in concordance between mono- and dizygotic twins can be ascribed to genetic causes.

Simple vs. complex disorders

Some genetic disorders are inherited as simple Mendelian traits. These are the monogenic disorders, which are caused by a single gene. Cystic fibrosis, mentioned above, is an example of a simple genetic disorder, where “simple”
Genetic disorders refers only to the predictability of the phenotypic outcome based on knowledge of the genetic constitution. The causative gene in cystic fibrosis, CFTR, codes for an ion transporter on the surface of epithelial cells (Gregory et al., 1990). Mutations at certain positions in the CFTR gene make the gene product malfunction. If an individual inherits one mutated copy of the gene from the father, and one from the mother, that individual will be a CF genotype carrier, who will eventually develop cystic fibrosis. Another example of a simple genetic disorder is hemophilia A, which is a blood disorder resulting in deficient blood clotting. This disease is caused by mutations in a single gene that encodes coagulation factor VIII, which is necessary for normal blood clotting (Toole et al., 1984). This disease occurs predominantly in males (1 in 5-10,000 births), since the gene resides on the X-chromosome. Females, who have two X-chromosomes, will almost always have one functional copy of the gene.

**Complex genetic disorders**

Most human genetic disorders are complex disorders, such as obesity, diabetes, certain types of cancer, and autoimmune disorders. These have been defined as “not exhibiting classic Mendelian inheritance caused by a single gene locus” (Lander and Schork, 1994). Complexities arise when the simple correspondence between genotype and phenotype breaks down. This complex behavior can occur due to one or more of the following reasons.

*Incomplete penetrance.* This means that carrying a disease predisposing genotype does not necessarily result in development of the disease. In other words, the disease predisposing gene may be necessary but not sufficient to develop disease.

*Genetic heterogeneity.* The same disease can result from a defect in any one of several genes. One can imagine that the end product in a biochemical pathway will be absent by blocking a reaction at any level in the reaction chain.

*Phenocopy.* Some individuals may develop a disease without carrying the predisposing allele.

*Polygenic inheritance.* In general, complex traits, including genetic disorders as well as normal physiological conditions are under the influence of many genes. Some genes may have a greater effect on the trait, while others have a smaller effect. The presence of several predisposing genes may be necessary to develop a disease. Genetic analysis of mice with insulin-dependent diabetes mellitus have, to date, revealed no less than 20 chromosomal regions harboring genes that affect the susceptibility to develop the disease (Cornall et al., 1991; de Gouyon et al., 1993; Denny et al., 1997; Ghosh et al., 1993; Ikekami et al., 1995; McAleer et al., 1995; Morahan et al., 1994; Podolin et al., 1997; Prochazka et al., 1987; Rogner et al., 2001; Serreze et al., 1994; Todd et al., 1991).
**Other transmission mechanisms.** The mitochondria, which carry a small circular genome of its own, are inherited only through the maternal germ line. Leigh syndrome, which gives rise to pathological changes in the brain, is a disorder caused by mutations in the mitochondrial genome (Rahman et al., 1996).

Genomic trinucleotide repeats, which expand through the generations, have been shown to be associated with Huntington’s disease. These repeats are located in the *Huntingtin* gene and form a part of the transcript. An increased number of repeats causes earlier disease onset. The pathological mechanism though, is still unknown (Andrew et al., 1993).

Genetic imprinting is another example of a type of genetic transmission that differs from traditional inheritance. After fertilization, some genes are subjected to imprinting, which causes expression from either the paternal or maternal allele to be silenced. The Prader-Willi syndrome is an example of a disorder that has been shown to be associated with imprinting. Loss of expression from paternally inherited genes in a region on chromosome 15, due to erroneous imprinting, is one cause of the disease (Nicholls et al., 1998).

**Conclusions “Genetic disorders”.**
Our genes are involved to a lesser or greater extent in regulation of most human disorders. Assessing the importance of a genetic component for a certain disease can be done by looking at the degree of familial aggregation, and concordance rates in siblings and twins. Most human disorders are considered complex. The factors listed above gives rise to complex inheritance patterns for many human disorders, and cause problems when performing genetic analyses in human populations. Some of these problems are circumvented by studying a restricted population, either with respect to a more narrow disease definition, or a more genetically homogenous population, such as ethnically or geographically isolated populations (e.g. Basques, Icelanders, or Finns).

**Benefits from using animal models**
The postulated genetic regulatory mechanisms of complex disorders, show that these disorders are not only difficult to study in humans, due to the presence of factors listed above, such as incomplete penetrance and heterogeneity, but also in animal models due to gene, and gene-environment interactions. The identification of these interactions is extremely difficult without the possibility of an experimental approach where it is possible to study interacting agents in a presumably controlled background, such as in an animal model.

Even if humans would be the best species in which to identify genomic regions involved in genetic disorders, the problem still remains of how to prove which genetic differences that are of importance. The confirmation that a
proposed alteration in the genome makes a difference to the course of pathogenesis requires an experimental approach where the effect of the predisposing alteration is either introduced onto an otherwise resistant genetic background, or replaced with a non-predisposing variant onto an otherwise susceptible background.

The use of animal models has proven to be a powerful approach to identify and understand genetic factors affecting the susceptibility and development of human disorders. The use of inbred strains of rats and mice makes it possible to avoid several of the inherent problems found in human genetic analyses. First of all, genetic heterogeneity is avoided. Secondly, the environment can be controlled. Also, modeling a disease in experimental animals gives an obvious advantage in that the genetic and environmental complexity can be manipulated.

There are animal models for a large number of human disorders. Examples of these are diabetes (Wicker et al., 1995), rheumatoid arthritis (Vingsbo et al., 1996), multiple sclerosis (Levine and Sowinski, 1973), obesity (Taylor and Phillips, 1996), hypertension (Kiprov, 1980), drug abuse (Berrettini et al., 1994), cancer (Vogel et al., 1999), and behavioral disorders (Reeves et al., 1995).

The arguments most often raised against the use of animal models to study human disorders are that the diseases are not comparable, i.e. that rodents and humans are too different to obtain biologically relevant results from rodent models of human disorders, and that most animal models require induction with something that triggers the development of disease. Even though the animal models display differences compared to the human form of disease, it is clear that there are also important parallels between them.

For example, an experimental model of rheumatoid arthritis, pristane-induced arthritis in rats (Vingsbo et al., 1996), fulfills the requirements needed for being diagnosed as RA (Table 1). It has been observed that autoimmune disorders often cluster in families, meaning that one disease is many times accompanied by other autoimmune diseases in these families. This would suggest that many of these disorders share common factors that increase the susceptibility (Becker et al., 1998; Jawaheer et al., 2001). The same has also been observed in some inbred rodent strains. Recent studies in animal models for autoimmune disorders have shown that there seem to be genetic factors that have a function in the development of autoimmunity as a whole, rather than in specific diseases (Bergsteinsdottir et al., 2000; Furuya et al., 2000; Jirholt et al., 1998; Kawahito et al., 1998). If these genetic factors could be identified in rodent models, it should be possible to find the corresponding factors, or the regulatory pathways where they exert their function, in humans.

**Genetic tools in animal research**
A lot of information on the genetic causes of a disorder can be obtained by cross breeding different strains of rats and mice. Typically, a disease susceptible
strain is crossed with a resistant strain. The offspring in the F1 generation become heterozygous for all autosomal loci throughout the genome, which means that they obtain one gene from each parent at all positions on the chromosomes. An incidence of disease in the F1 generation indicates the involvement of dominant disease genes, meaning that one copy of a disease gene is sufficient for the development of disease. The incidence in the F1 generation also provides some information on the penetrance of the disease. If only a portion of the progeny gets affected, it would indicate a reduced penetrance of the disease genes, since all F1 progeny are genetically identical, except for the sex chromosomes. The next step in the breeding is to either cross F1 progeny to other F1 progeny to produce F2 intercross progeny, or to cross F1 progeny to one of the parental strains to produce F2 backcross progeny. The illustration below outlines the different types of crosses discussed here (Figure 1).

Figure 1. *Breeding scheme to obtain BC2 backcross and F2 intercross progeny*

The progeny in the F2 generation will have any combinations of genotypes throughout the genome. Any given locus may display homozygosity for alleles from either of the parental strains. The progeny from the F2 generation can be used to search for genetic markers that co-segregate with disease, in order to identify genomic regions that contain genes involved in disease development. This is further discussed in the chapter on quantitative trait locus analysis below.
Congenic strain
If one has already focused on an interesting genomic region, there are other
types of genetic tools that can be applied to experimental animals to test the
phenotypic effect exerted by genes in the region. One such tool is the breeding
of congenic strains. Suppose that a region has been identified as being
associated with a particular trait, and further, that this region originates from a
susceptible strain of rats/mice. It is highly possible that additional regions, in
the genome of the susceptible strain regulate the trait under study. It would
then be of interest to isolate the effect that this particular region has on the
trait. By congenic breeding, one can isolate the genomic region on a different
genetic background, by crossing the susceptible strain with a resistant strain,
followed by crossing the progeny back to the resistant strain multiple times. For
each generation of backcrossing, the genome of the progeny is enriched for
genes from the resistant strain. For each round of backcrossing, the interesting
region is typed with one or more genetic markers, to ensure that alleles from
the susceptible strain are retained. After 12 generations of backcrossing it can
be assumed that the genome contains almost only alleles from the resistant
strain, except for the region that has been selected for, which is heterozygous at
this stage. An F1 intercross produces offspring that become homozygous for the
target region. Since it is time consuming and expensive to breed animals for
this many generations, a more effective breeding technique is now commonly
used. This technique, named “marker assisted selection protocols” (MASPs),
depends on the screening of progeny with genetic markers throughout the
geno, in order to select the offspring that show the highest rate of
background genome to be founders of the subsequent generation (Wakeland et
al., 1997). This limits the time needed to obtain a clean congenic strain to
approximately 5 generations of breeding. In a recent study on a mouse model
for SLE (systemic lupus erythematosus), Morel et.al. were able to show that a
previously identified susceptibility locus, Sle1, in fact contained three loci,
which could independently cause the disease phenotype. The fine mapping of
the Sle1 region was performed through the analysis of congenic strains, which
made it possible to reveal this cluster of functionally related loci (Morel et al.,
2001).

Recombinant inbred strains
A similar, but more random, approach is to generate recombinant inbred (RI)
strains. The genomes of these strains consist of random homozygous fragments
from two different strains. A number of different RI strains can be a powerful
tool to identify and isolate genomic regions affecting a trait, since a locus of
interest can be narrowed down by comparing recombination events in them.

Advanced intercrossed lines
F2 intercross progeny display a limited number of recombination events, which
results in broad confidence intervals (CIs) for quantitative trait loci. These fairly
large chromosomal regions are not suitable for positional cloning or for candidate gene identification. Advanced intercross lines (AIL) offer a possibility to facilitate high-resolution mapping of these QTLs. Advanced intercross lines are produced by random intercrossing of progeny, starting in the F2 generation. For each generation, the genome of the progeny is successively enriched in recombination events (Darvasi and Soller, 1995). In this way, large genomic fragments, inherited from one of the parental strains, are split up to contain smaller fragments from both founder strains. This could be compared to the shuffling of cards. Imagine two decks of cards with different colors. For each turn of shuffling the chance will increase of finding two adjacent cards with different colors. The AILs can be powerful in simultaneously limiting the confidence intervals for several QTLs, which makes this method advantageous over congenic strains. Also, this method can make it possible to dissect QTLs with broad CIs into discrete regions that may contain more than one QTL.

Transgenic strains
In mice and rats, it is possible to introduce single genes or genomic fragments by molecular genetic techniques, in order to produce transgenic animals. The introduced genetic material can originate from a different strain or even from a different species. This method involves the introduction of pure DNA by microinjection into the pronuclei of fertilized eggs. If the introduced DNA is stably incorporated into the genome it will be propagated to all cells in the developing animal (Palmiter et al., 1982). In a study of gene function by targeted deletions of genomic fragments in mice, Zhu et al. were able to identify a novel gene involved in the regulation of triglyceride production by the use of the transgene technique (Zhu et al., 2000). A deletion of a 450kb genomic region on mouse chromosome 11 caused the animals to suffer from hypertriglyceridemia, liver and heart enlargement, growth retardation, and shortened life span. To identify the genes responsible, transgene complementation was used. Four mouse BACs (bacterial artificial chromosomes), covering the deleted region, and two human YACs (yeast artificial chromosomes) containing sequences orthologous to the deleted region, were used to create transgenic mice. It turned out that one of the transgenic animals, containing one of the human YACs, was indistinguishable from wildtype mice. Further analysis of the sequence of this human YAC, together with expression analysis, revealed that the absence of the gene OCTN2 was responsible for the phenotype observed in mice that were homozygous for the deletion.

Knock-out strains and conditional knock-out strains
With mice, but not with rats, it is possible to create knockout animals, where the expression of a targeted gene or chromosomal region has been silenced. Knock-out mice are produced by growing embryonic stem cells in culture, creating targeted mutations in them and subsequently transplanting them into
pre-implantation mouse embryos to produce a chimera (te Riele et al., 1992; Thomas and Capecchi, 1987). This embryo will comprise wild-type cells as well as lineages derived from the mutant cells. If the chimera has functional germ cells that are derived from the mutated cells, offspring can be bred from the chimeras to produce heterozygous or homozygous mutant mice. This does not work in other animals, since stem cells capable of producing germ line chimeras have so far not been reproducibly isolated except from certain strains of mice.

Another, similar approach takes advantage of the P1 bacteriophage derived Cre recombinase, that catalyses recombination between specific recognition sites, called loxP (Araki et al., 1995; Hamilton and Abremski, 1984). The introduction of loxP sites around a target sequence causes a recombination between these sites, in the presence of Cre recombinase, which results in excision or reversion of the target sequence. These methods can be very powerful in studies of gene function. Two recent studies independently reported on the importance of a growth factor for normal limb bud development, which was studied using the Cre-LoxP system (Lewandoski et al., 2000; Moon and Capecchi, 2000). Because the absence of this growth factor causes early embryonic lethality, it had not been feasible to study the function of this gene in vivo without a conditional system for silencing of the expression, offered by the Cre-loxP method.

The mouse or the rat?
Historically, the mouse has been used extensively in genetic research. For this reason, the pace at which genetic resources have been developed have been higher for the mouse than the rat. This includes genetic marker maps (Dietrich et al., 1996; Dietrich et al., 1994; Rhodes et al., 1998), a radiation hybrid map (Van Etten et al., 1999), YAC, BAC, and PAC genomic libraries (Chartier et al., 1992; Haldi et al., 1996; Osoegawa et al., 2000), and the whole mouse genome sequence (Celera Inc. 2001). However, during the past five years, high-throughput technologies for genotyping and sequencing have reduced the lead that mouse genetic resources have had over the rat. Most resources that are available for the mouse are now also available for the rat, with the exception for the large-scale sequencing of the rat genome, and the possibility to create knock-out rats, as mentioned above. On the other hand, the rat has traditionally been used in research on mammalian physiology, mainly due to its comparatively larger size. As a result of this research, a wide variety of rat strains have been selected for their expression of traits related to complex diseases, such as diabetes, arthritis, multiple sclerosis, renal disease, hematological disorders, hypertension, autoimmune disorders, eye disorders, and behavioral disorders. With the development of genetic tools for the rat, these models have become an invaluable asset for the identification the genetic components that regulate complex disorders. The recent advances in genetic and physical mapping of the mouse, rat, and man have created opportunities
for the construction of comparative maps (Nilsson et al., 2001; Remmers et al., 1999; Serikawa et al., 1999; Watanabe et al., 1999). These could make it feasible to ‘jump’ between the species, if the desired methodology is not accessible for the organism.

**Conclusions “Benefits from using animal models”**

Unarguably, animal models for human disorders display differences compared to the human form of the disorders. But, focusing the interest on their similarities and on the possibility to apply a wide set of genetic tools on the experimental animals, make animal models an invaluable, and probably necessary, instrument to understand the genetic basis of complex disorders.
Autoimmunity

A group of complex disorders that display genetic predisposition are the autoimmune diseases. Examples of autoimmune diseases are systemic lupus erythematosus (SLE), Hashimoto’s thyroiditis, multiple sclerosis, insulin-dependent diabetes mellitus and rheumatoid arthritis (RA). These are characterized by incorrect self-recognition, which leads to immune reactions against either specific organs or non-organ specific targets. It has been reported that self-reactivity is a normal occurrence in the immune system, which suggests an essential role for this behavior, but also that the self-response is kept in control under normal conditions (McDevitt and Wakeland, 1998).

RA – Brief description and genetic components

Rheumatoid arthritis is an autoimmune disorder, which causes chronic inflammation in the small joints of the hands and feet. The criteria for being diagnosed with RA are shown in table 1.

The etiology and environmental factors that contribute to the risk of developing RA are poorly understood. It has been proposed from epidemiological studies that coffee consumption (Heliovaara et al., 2000), smoking (Wolfe, 2000), oral contraceptives (Brennan et al., 1997), and high serum cholesterol levels (Heliovaara et al., 1996) are associated with an increased risk of developing RA. An infectious etiology of rheumatoid arthritis has been postulated for a long time, but so far none has been identified. The prevalence, or population relative frequency, of RA is approximately 1% in Caucasian populations. Women are affected three to four times more frequently than men. RA displays familial aggregation with a sibling relative risk, $\lambda_s = 8$ (Wordsworth, 1995). This means that, given one affected sibling in a sibling pair, the risk of a second sibling being affected is 8 times larger than for individuals in the population on average. The concordance rate for monozygotic twins has been estimated to 15% (Silman et al., 1993). In a recent study of twins from Finland and the United Kingdom, the heratibility, or the variation in liability to disease accounted for by genetic variation, was estimated to 60% (MacGregor et al., 2000). Taken together, these data show that genetic factors provide a substantial contribution to RA in the population.

Efforts made to identify genetic factors that explain the susceptibility to RA have to date come up with only modest results. A strong association between the HLA region and RA has clearly been shown (Cornelis et al., 1998; Stastny, 1978).
Table 1. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis (Arnett et al., 1988). Four of these criteria should be fulfilled to be diagnosed with RA.  
Abbreviations: PIP = proximal interphalangeal joint, MCP = metacarpophalangeal joint, MTP = metatarsophalangeal joint.

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<td>1. Morning stiffness</td>
<td>Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement.</td>
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<td>2. Arthritis of 3 or more joint areas</td>
<td>At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow, knee, ankle, and MTP joints.</td>
</tr>
<tr>
<td>3. Arthritis of hand</td>
<td>At least 1 area swollen (as defined above) joints in a wrist, MCP, or PIP joint.</td>
</tr>
<tr>
<td>4. Symmetric arthritis</td>
<td>Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry).</td>
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<tr>
<td>5. Rheumatoid nodules</td>
<td>Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician.</td>
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<tr>
<td>6. Serum rheumatoid factor</td>
<td>Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in ~5% of normal control subjects.</td>
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<td>7. Radiographic changes</td>
<td>Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify).</td>
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It has been proposed that the association to MHC in autoimmune diseases is caused by the escape of self reactive T cells from thymic selection due to poor self-binding properties of disease associated MHC class II molecules (Ridgway et al., 1999).

Two genome-scans of rheumatoid arthritis families have been published (Cornelis et al., 1998; Jawaheer et al., 2001). The sib-pair analysis performed by Cornelis et al. revealed HLA as a susceptibility locus. They could also identify fourteen loci that did not reach the threshold for suggestive linkage. In a more recent study on sib-pairs, Jawaheer et al. could identify six susceptibility loci residing on chromosomes 1, 4, 6 (HLA), 12, 16, and 17, respectively. None of these loci, however, except for the HLA locus, reached the threshold for significant linkage. The locus on chromosome 17 reached the level of a suggestive linkage.
Induced arthritis in animal models
A number of rodent models of RA exist. These have the fact that the disease is induced in common. There are a variety of induction methods, of which the most commonly used is heterologous or homologous type II collagen, a component of cartilage. The introduction of a joint-derived antigen triggers an autoimmune response against peripheral joints. Another method for induction of arthritis in rodents is the use of an adjuvant, such as mycobacterium tuberculosis in mineral oil (Pearson, 1956), avridine in mineral oil (Vingsbo et al., 1995), pristane (Vingsbo et al., 1996), or Freund’s mineral oil (Holmdahl and Kvick, 1992). The adjuvants, which are considered to be non-antigenic, are believed to enhance a nonspecific immune response that somehow triggers an auto-reactive immune attack on the peripheral joints of the paws.

Conclusions “Induced arthritis in rats”
Animal models of rheumatoid arthritis can provide important clues as to the causes and regulatory mechanisms of the human form of disease. There are probably species-specific differences between the human and rodent forms of the disease, but also important parallels that can be used to understand the etiology and development of the disease and to provide the basis for establishing novel therapies.
**QTL analysis**

Quantitative trait loci (QTL) analysis is a method to identify chromosomal regions that affect the variability of a trait without prior knowledge of the genetic causes of the observed trait variation.

**Genetic maps**

QTL analysis requires genetic maps, which are representations of genetic markers positioned within genomic regions. See figure 2 below. The distances between the markers are determined by the frequency of recombination between them. This represents a statistical distance measured in units of centimorgan (cM), i.e. the probability of a recombination event between the markers, rather than a physical distance measured in number of nucleotides.

There are different types of genetic markers. The most commonly used are the RFLP (restriction fragment length polymorphisms), SSLP (simple sequence length polymorphisms), and SNP (single nucleotide polymorphisms) markers. They can be scored, either for size-differences between individuals, or for DNA sequence differences. This makes it possible to determine the marker genotype of an individual.

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Figure 2. A genetic map of rat chromosome 4 with 31 microsatellite markers positioned along the chromosome.

![Genetic Map of Rat Chromosome 4](image-url)
QTL mapping
To detect linkage between a marker and a putative QTL, one must have a segregating population, such as progeny from an experimental animal cross.

Figure 3. The illustration briefly outlines the principal of QTL analysis. In practice, computer programs designed for this purpose perform the statistical calculations used for testing the strength of the evidence for the presence of a QTL adjacent to a genetic marker.

**Rationale of QTL Analysis**

Assume that the possible marker genotypes at a chromosomal locus are AA, or AB.

To detect a QTL that is linked to a genetic marker, one compares the mean trait values for the group with genotype AA to the group with genotype AB.

\[
\text{Difference} = \text{Mean}_{AA} - \text{Mean}_{AB}
\]

The difference in mean trait values provides an estimate of the phenotypic effect of substituting a B allele for an A allele.

\[H_0 : \text{Difference} = 0\]
\[H_A : \text{Difference} \neq 0\]

The presence of a QTL cannot be ruled out if the difference in mean trait values between the two groups are significantly different from zero.
Interval mapping is a commonly used method for detecting QTLs (Lander and Botstein, 1989). The principle behind interval mapping is to test a model for the presence of a QTL between two mapped marker loci at many positions. The strength of a given model is tested using the maximum likelihood method, measured as the likelihood ratio (LR) or logarithm of the odds (LOD). Maximum likelihood involves searching for QTL parameters that give the best estimation for the quantitative trait distributions that are observed over the possible genotypic groups of a putative QTL. The models are evaluated by computing the likelihood of the observed distributions with and without fitting a QTL effect to them. The LOD score is the logarithm of the ratio between these likelihood estimates.

Variations on the same theme include Composite Interval Mapping (CIM) (Zeng, 1994), and Multiple Interval Mapping (MIM) (Kao et al., 1999). The CIM method makes use of a combination of interval mapping and multiple regression. By using information from markers outside a defined interval, one can avoid other QTLs affecting the test results within that interval. The MIM method allows for simultaneous scanning of multiple putative QTLs by the joint analysis of multiple marker intervals.

The results obtained from QTL analysis provide information on the location of a putative QTL and the statistical strength of the presence of a QTL in that location, see figure 4.

Figure 4. Graphical illustration of results from a QTL scan of a genomic region. The log likelihood for the presence of a QTL has been plotted against the genetic distance. The width of the shaded area represents the 95% confidence interval for the QTL, which comprises about 25 cM.
The most commonly used experimental animal populations for QTL analysis are F2 intercross and BC2 backcross progeny. In these experiments, QTL mapping gives a very crude measurement of the QTL position, with a typical confidence interval of 10-30cM. This genetic distance translates into a physical distance of approximately 10-30 million basepairs, which could harbor hundreds or even thousands of genes.

**Phenotypes in QTL analysis**

Since QTL analysis is dependent on the measurement of a phenotype, it is important to consider the nature of these quantitative traits. For many of the commonly used QTL software packages, such as Mapmaker/QTL (Lincoln et al., 1992) and QTL cartographer (Baston et al., 1994), the test statistics assume the traits to be normally distributed. Deviations of the trait distribution from normality may result in false positive results or an erroneous estimation of the phenotypic effects of QTLs, or even failure to identify existing QTLs. This can, to some extent, be overcome by variable transformation, whereby the distribution of trait values can be approximated to a normal distribution.

Many times, a scoring system such as disease severity is used for the quantification of a trait. The scoring system measures severity on an interval scale. If the underlying trait variable is considered normally distributed, the scoring system poses no problem in the analysis, apart from reduced statistical power. If, on the other hand, it is not possible to determine the distribution of the underlying variable, one has to turn to other test methods, such as non-parametric analyses that do not have a prerequisite for a defined trait distribution. Kruglyak & Lander (1995) have developed a QTL analysis method that can handle non-parametric traits. However, this method has only been implemented for sib-pair analysis at this point.

Multiple traits that show a strong correlation between them will, by necessity, show association to the same QTL. This does not necessarily mean that the underlying gene/gene in the QTL have a causative effect on all traits, or even for any of the traits studied. The variation observed for the correlated traits may be a secondary effect caused by the effect that the gene/gene has on a primary causative trait. The observation that a phenotype under study is associated to a certain QTL may therefore lead to erroneous conclusions about the genetic regulation of that phenotype.

**Significance**

What significance level should be chosen in order to be confident that a linked locus contains a true QTL? There is no definite answer to this question. The conventional confidence level used in a genome scan is 95%. This means that the probability of a false positive result is 5%. When performing QTL analysis using interval mapping, or variants thereof, these five percent must be translated into the corresponding logarithm of the odds ratio (LOD). This value will vary, depending on the type of experimental cross that is being used.
Lander & Kruglyak (1995) have proposed a set of significance thresholds for various conditions that, for a long time, have been used as a standard for reporting linkage results. These proposed significance levels, however, assumes the use of an infinitely dense map and traits that are normally distributed.

To establish a meaningful significance level in cases where the trait distribution deviates from normality, one can perform permutation tests on the data. Permutation testing is a method that results in an experiment-wise significance level. E.g. in an experimental animal cross where a genetic map has been established, the trait values are shuffled randomly between the individuals to break any relation between genotype and phenotype. For each turn of shuffling, a full QTL analysis is performed and the highest test statistic recorded. This is subsequently repeated many times ($10^3 - 10^4$). The resulting distribution of maximum test statistics shows what level of the test statistic that yields, for example, a genome-wide nominal significance level of $\alpha=0.05$. If the genetic map contains gaps, the permutation test method can easily result in an overestimation of the test statistic needed for $\alpha=0.05$. This is due to the fact that a gap in the genetic map results in a high probability of recombination events in the gap, which in turn increases the probability of fitting a QTL in that region. If the genetic map suffers from large gaps, it could be advisable to instead split these regions in to separate linkage groups before performing permutation tests to obtain an experiment-wise significance level.

Multiple testing is another factor to consider in choosing an appropriate significance level. A common way to treat situations when multiple testing have been performed is to apply some kind of correction to the results in order to attain a nominal significance level of e.g. $\alpha=0.05$. The Bon-Ferroni correction, which is commonly used states that an independent test is considered significant if the probability of a given outcome is less than $\alpha/n$, where ‘n’ is the number of independent tests performed.

**Conclusions “Quantitative trait loci analysis”**

QTL analysis is a statistical method used to detect chromosomal regions that show association to a quantitative phenotype. The results from such analyses can provide an approximate location of a genomic region harboring a gene/genes, which could be directly or indirectly responsible for the variability of the phenotype. In performing a QTL analysis one has to consider factors that can affect the results, such as, density of genetic markers, distribution of the phenotype, multiple tests performed, and level of significance.
PRESENT INVESTIGATION

Aim

The aim of this study was to identify and characterize genetic components that affect the susceptibility to rheumatoid arthritis in animal models.

Introduction

Pristane-induced arthritis in rats is an animal model for rheumatoid arthritis (RA) (Vingsbo et al., 1996). Pristane is a natural component in plants, as a part of the chlorophyll, and is therefore ingested and also absorbed by animals (Garrett et al., 1989). However, some strains of rats develop a disease that mimics many aspects of RA following a subcutaneous injection with pristane. Rats develop the disease a few weeks after induction. The initial manifestation is a sudden inflammation in a peripheral joint whereafter the disease spreads to other peripheral joints and usually develops along an active chronic disease course. The inflammation is restricted to the peripheral joints and does seldom affect other more centrally located joints or other tissues. As in RA, the joints are usually affected symmetrically.

Genetic predisposition to pristane-induced arthritis

There is a genetic predisposition to the development of PIA. DA and Lew.1f rats are highly susceptible, while E3 rats are almost resistant. Studies using MHC-congenic Lew rat strains have shown that the MHC locus contributes to the genetic predisposition (Vingsbo et al., 1996). Interestingly, this effect was seen only on the chronic development of disease, whereas no significant association to the time of onset or the incidence was observed.

However, the influence of the MHC cannot explain the dramatic difference in susceptibility to PIA seen between DA and E3, as the MHC haplotypes of these strains are equally permissive on a Lew background. Instead, there is a major non-MHC genetic influence that determines the difference in PIA susceptibility observed between the DA and E3 strains. PIA is clearly a polygenic disease, as the penetrance of the disease is variable in a series of recombinant inbred strains made from DA and E3 (Vingsbo et al., 1996; Vingsbo-Lundberg et al., 1998).

The DA strain is highly susceptible to PIA and develops the disease with high incidence, pronounced joint erosions, high clinical severity and with a chronic relapsing disease course. The Lew.1f strain is also highly susceptible, but displays less erosion of bone and cartilage during the acute and chronic phase of disease compared to the DA strain. In contrast, the E3 strain is almost resistant. Among recombinant inbred strains from DA and E3, the DXEA strain...
develops mild disease with high incidence, the DXEB strain has more severe and chronic disease but with lower incidence whereas the DXEC strain is almost resistant (Vingsbo et al., 1996). This demonstrates that different genes control different disease phenotypes. The disease development and progression in the strains used is summarized in table 2:

Table 2. Development and progression of pristane induced arthritis in different rat strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>n</th>
<th>Mean day of onset</th>
<th>Mean max clinical score</th>
<th>Frequency of arthritis</th>
<th>Frequency of chronic arthritis</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA</td>
<td>17</td>
<td>14 ± 2</td>
<td>11</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>E3</td>
<td>23</td>
<td>38 ± 6</td>
<td>2</td>
<td>9%</td>
<td>0%</td>
</tr>
<tr>
<td>Lew.1F</td>
<td>10</td>
<td>17 ± 4</td>
<td>12</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>DXEA</td>
<td>20</td>
<td>31 ± 21</td>
<td>3</td>
<td>70%</td>
<td>10%</td>
</tr>
<tr>
<td>DXEB</td>
<td>24</td>
<td>72 ± 34</td>
<td>5</td>
<td>33%</td>
<td>30%</td>
</tr>
<tr>
<td>DXEC</td>
<td>20</td>
<td>32 ± 15</td>
<td>3</td>
<td>10%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Results

Different genes control different phases of the disease in a model for rheumatoid arthritis in rats (Paper I)

In this paper, we analyzed the progeny from a cross between the resistant E3 strain and the susceptible DA strain. The aim of this study was to identify genetic regions that could explain the difference in susceptibility to pristane-induced arthritis observed between these strains. The QTL analysis identified five autosomal loci that affected the susceptibility to pristane-induced arthritis.

The development of pristane-induced arthritis can be viewed as having three phases. Firstly, there is the onset phase of disease, which is characterized by an initial acute inflammation. This is followed by the second phase, which exhibits a prolonged severe inflammation with bone and cartilage erosion. Eventually, the disease enters a chronic phase with continuing inflammation and progressive erosion/healing of bone and cartilage, which leads to improper bone formation, and thus malformation of the joints.

We found that the five identified loci were exclusively associated with a certain phase of the disease (table 3).
Table 3. QTLs identified in the (E3xDA)f2 intercross

<table>
<thead>
<tr>
<th>QTL</th>
<th>Chr</th>
<th>Trait</th>
<th>Marker</th>
<th>Inheritance</th>
<th>LOD</th>
<th>Variation explained</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Regions associated with arthritis onset</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pia2</td>
<td>4</td>
<td>Day of onset</td>
<td>D4Agh14</td>
<td>E3 dominant</td>
<td>3.9</td>
<td>32 %</td>
</tr>
<tr>
<td>Pia3</td>
<td>6</td>
<td>Day of onset</td>
<td>D6Wox5</td>
<td>E3 dominant</td>
<td>4.5</td>
<td>25 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Regions associated with arthritis severity and early joint erosion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pia4</td>
<td>12</td>
<td>No. of affected paws day 28</td>
<td>D12Wox14</td>
<td>DA additive</td>
<td>8.4</td>
<td>22 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Regions associated with arthritis chronicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pia5</td>
<td>4</td>
<td>Inflammation score day 120</td>
<td>D4Wox20</td>
<td>DA additive</td>
<td>4.5</td>
<td>24 %</td>
</tr>
<tr>
<td>Pia6</td>
<td>14</td>
<td>No. of affected paws day 83</td>
<td>Csna</td>
<td>DA recessive</td>
<td>4.9</td>
<td>19 %</td>
</tr>
</tbody>
</table>

a The effect of Pia2 was detected in female rats only (corresponding LOD score in male rats, 0.03).
b The effect of Pia5 was detected in male rats only (corresponding LOD score in female rats, 0.2).

Two QTLs were associated with the onset of disease, Pia2 and Pia3, located on chromosome 4 and 6, respectively. Surprisingly, the disease promoting alleles at these loci originated from the resistant E3 strain. Alternatively, a protective effect was exerted by the corresponding DA alleles. The earlier onset that was caused by Pia2 was only observed in female rats. On chromosome 12 we identified a locus, Pia4 that showed a strong association to disease severity. Two QTLs, Pia5 and Pia6, were exclusively associated with severity during the chronic phase of disease. Pia5, located on chromosome 4, was associated with histological scoring of inflammation at day 120. This effect was only observed in male rats. Pia6, on chromosome 14 was associated with chronic severity. This association peaked at day 83 after induction with pristane.

These findings suggest that different sets of genes in the rat genome control different events during disease development and further, that some loci exert their effect in a gender-specific manner.

Complex genetic control in a rat model for rheumatoid arthritis (Paper II)

In this study, a cross between the susceptible strain DA and a resistant recombinant inbred (RI) strain, DXEC, was analyzed. Despite carrying the severity locus Pia4 on chromosome 12 and the chronicity locus Pia6 on chromosome 14, the DXEC strain was found to be almost resistant to pristane-induced arthritis. The aim of this experiment was to investigate whether new loci that confer susceptibility could be identified in a cross where two important disease predisposing loci were shared in all progeny.

The RI strain DXEC originated from a cross between DA and E3. Consequently, its genome is a mixture of fragments from these two parental
Results

strains. An RI strain is defined as being homozygous for all loci, but these can originate from either of the founder strains. Crossing the DA strain and the DXEC strain results in enrichment for DA alleles, since both strains contribute these alleles.

In this cross, we could confirm the onset locus, *Pia3*, on chromosome 6. As was observed previously, this locus contains a gene/genes that causes earlier disease onset if at least one allele is inherited from the E3 strain. A puzzling finding in this cross, was that *Pia3* only showed an effect in males, whereas it was equally permissive in males and females in the (E3xDA)F2 cross.

Two new loci were identified in this cross. One locus on chromosome 1, *Pia8*, was associated with disease severity. The allele that conferred susceptibility originated from the resistant E3 strain and the effect was found to be restricted to female rats. The second locus, *Pia7*, was located on chromosome 4 and it was associated with severity during the onset phase of disease. The underlying gene/genes was found to have an effect in rats homozygous for the DA allele at this locus.

We could also detect a suggestive linkage to the MHC region on chromosome 20. This locus was associated with severity at day 56. This coincides with the time at which the disease usually progresses into the chronic phase. The justification for stating that the chronic phase starts at this time is that animals affected at this point, in the majority of cases, remain affected.

There is a significant difference in severity (severity score day 35) between offspring from the two crosses (E3xDA)F2 and (DAxDXEC)f2. This difference could be explained by the presence of *Pia4* in all progeny from the latter cross.

We did not find any evidence for an effect of the newly identified QTLs in the previous cross, (E3xDA)F2. There could be several reasons for this. Other research groups have reported the identification of new QTLs after stratifying their data with respect to a permissive genotype at a susceptibility locus (Remmers et al., 1996). One can imagine two loci that have an effect on a certain trait and further, that one locus shows a much stronger association with the trait than the other. In this case, the variation caused by the second locus could be “hidden”, or “masked” by the effect from the first, stronger, locus. If, on the other hand, one performs the analysis on a subset of data that contains only animals with the permissive genotype at the first locus, the remaining trait variability cannot be due to the first locus and is therefore caused by the second locus.

Another possible explanation as to why we did not detect these loci in the previous cross, could be the presence of epistasis. Per definition, epistasis is the influence of alleles at one locus on the phenotypic effects of alleles at other loci (Bateson, 1909). It thus reflects the fact that the expression of genetic variation is under the influence of other genes.

Thus, the reasons why we could not identify these new loci in the (E3xDA)F2 cross could be, either that a QTL with a large effect “masked” the
smaller effect caused by the new QTLs or that the new QTLs are dependent on certain alleles at another locus (i.e. epistasis). To test for these possibilities, we re-analyzed the (E3xDA)F2 cross. This was performed by stratifying the data with respect to the genotype at loci that are DA homozygous in the DXEC strain. In this way we tried to mimic the conditions of the (DAxDXEC)F2 cross. In the re-analysis of the (E3xDA)F2 cross we used, for a certain locus, only those animals which had been scored as DA homozygous. This stratification was then performed for all loci where the alleles were derived from the DA strain, in the DXEC strain. The expected outcome, if there was a “masking”-effect, would be that we could detect the new QTLs in the (E3xDA)F2 cross, given that we select for DA homozygosity at one of the QTLs identified earlier. Alternatively, if epistasis was the cause, we would expect to identify the new QTLs, given that all animals share a certain locus with alleles derived from the DA strain, but in itself did not act as a QTL.

The analysis revealed that the loci Pia1 and Pia7 could be identified in the (E3xDA)F2 cross and that the effect of these loci were dependent on DA homozygosity at other loci on chromosome 14 and 18. These regulatory loci did not, in themselves, have any detectable effect. However, they were a prerequisite for Pia1 and Pia7 in order to exert an effect on the trait variation. This would suggest the presence of epistasis. The number of animals left after stratification was 17-20, which is a very low number for performing the analysis. The multiple tests performed in the analysis should also be considered. Still, these QTLs show association to the same traits and with the same inheritance pattern as in the (DAxDXEC)F2 cross.

In summary, these findings show that additional loci can be identified if the effects of other loci are neutralized. Also, results from this study suggest that epistasis may have an important role in regulating the development of pristane-induced arthritis.

Genetic links between the acute phase response and arthritis development in rats (Paper III)

Previously, it had been observed that the levels of interleukin-6 (IL6) were different between DA and E3 rats, with regards to pristane-induced arthritis. This study was performed in order to investigate the genetic regulation of IL-6 and the related cytokines and their potential involvement in the regulation of pristane-induced arthritis.

The acute phase response is marked by an instant increase of certain serum proteins upon infection and inflammation, which are the acute phase proteins (Peri and Rinaldi, 1998). The measurement of the acute phase proteins and the components associated with the cytokine system may provide valuable information about the initial processes in arthritis development. The levels of α1-acid glycoprotein (AGP), α1-inhibitor (α1-I) and interleukin-6 (IL6), were determined in the F2 progeny from a cross between the rat strains DA and E3.
These traits were used in QTL analysis as to identify loci explaining their phenotypic variability.

The results revealed that QTLs, which explains a portion of the variability observed from the acute phase proteins, were partly co-localized with regions identified previously to control arthritis development. The arthritis severity locus, *Pia4*, on chromosome 12 (Vingsbo-Lundberg et al., 1998) was found to be associated with both AGP (measured at day 14) and IL6 (measured at day 35). These associations could only be detected in male rats. Interleukin-6 (measured at day 14) was found to be associated with a locus on chromosome 14. This QTL overlaps with *Pia6*, which is associated with severity during the chronic phase of the disease. The effect of this QTL on levels of IL6 could only be detected in male rats, as well. Also, we identified a suggestive QTL, which explained parts of the observed variability for AGP (measured at day 14) that coincided with *Ciaa3*, a locus on the rat chromosome 1, which is reported to be associated with anti-collagen antibody titers (Griffiths et al., 2000).

Thus, the question arises, are the observed correlations causative or secondary effects? It has been reported that there is an association between acute phase proteins and arthritis development (Charles et al., 1999; O’Hara et al., 2000; Peri and Rinaldi, 1998). The results presented in this paper show that there is an association between certain acute phase proteins and different disease parameters of pristane-induced arthritis in rats. Furthermore, that the chromosomal regions contain genes, which could directly or indirectly regulate the levels of these proteins. These regions coincide with regions identified previously that are involved in controlling the susceptibility to pristane-induced arthritis. This does not necessarily mean that the same genes are operating on both traits, even though it is a compelling thought. Regions that coincide could argue for the presence of gene clusters, where the individual genes have in common the fact that their products are parts of the same biochemical/physiological pathways (1999; Morel et al., 2001). This could explain why so many of the autoimmune QTLs seem to be co-localized in the genome.

Identification of two novel non-MHC loci which control pristane-induced arthritis in rats (Paper IV)

A cross between the pristane-induced resistant rat strain E3 and the susceptible Lew.1f strain was set up to confirm previous findings from the crosses between the DA and E3 strains. Also, we wanted to map a susceptibility locus that co-segregates with the albino phenotype of Lew.1f, which is caused by alleles at the C-locus on chromosome 1. The development of pristane-induced arthritis in Lew.1f rats differ somewhat from what is seen in DA rats, in that they show less erosion of bone and cartilage during the acute and chronic phase of the disease.

The disease development in the (E3xLew.1f)F1 population displayed a reduced incidence and severity, as compared to the Lew.1f strain. Interestingly,
only female rats developed the disease in the F1 population, which further confirms the observation that there is a strong gender bias in this disease.

In this cross we could identify two new QTLs on chromosomes 1 (Pia9) and 16 (Pia11), respectively. Also, we found a suggestive linkage to chromosome 14. The locus Pia4 identified previously, on chromosome 12, was confirmed in this cross with the same inheritance pattern as in the (E3xDA)F2 cross.

In the same region as the C-locus, which is responsible for the albino phenotype of the Lew.1f strain, we identified the locus, Pia9, which was associated with arthritis severity during the acute phase of the disease. The predisposing allele originated from the Lew.1f strain. This was also confirmed in a congenic strain, where E3 alleles in the Pia9 region on a Lew.1f background, had a significant protective effect. Also, the Pia11 locus was associated with arthritis severity in the acute phase of the disease, but with the predisposing allele derived from the resistant E3 strain.

A locus, Pia3, which was identified previously in the E3xDA cross, did not show any effect in the E3xLew.1f cross. This locus was associated with disease onset, where the E3 allele in a dominant mode caused an earlier disease onset. The fact that no effect of this locus could be detected in the present cross, would suggest that alleles from the susceptible strain DA, at the Pia3 locus, confer a protective effect, causing a later onset of disease.

Taken together, these findings suggest that different strains carry unique predisposing disease loci, as well as loci that are common between different strains.

An improved genetic map of the rat (Paper V)

In order to locate integration points between three major genetic maps of the rat genome (Bihoreau et al., 1997; Brown et al., 1998; Jacob et al., 1995), a high-resolution linkage map was constructed from various crosses between the rat strains DA and E3. The genetic map is based upon genotype data from two separate projects. First, the rat arthritis project which consists of three crosses, (DAxE3)F2, (E3xDA)F2 and (DAxDXEC)F2, where DXEC is a recombinant inbred strain derived from a cross between the DA and E3 strains. Second, the rat experimental autoimmune encephalomyelitis project, which consists of one cross, (E3xDA)F2. Together, these crosses comprise a total of 600 F2 progeny. A set of microsatellite markers has been screened for size polymorphisms in the parental strains, of which approximately 320 have shown to be informative.

The size of the rat genome is approximately 2000 cM. Assuming one crossover per 100 meioses, the information from 600 F2 progeny gives a map resolution making it possible to order markers that are separated by a genetic distance above 0.2 cM.

This map was also used to characterize four recombinant inbred (RI) strains, DXEA, DXEB, DXEC and DXER, all derived from the cross E3xDA. The
strain DXER turned out to carry microsatellites of sizes that were not consistent with either of the parental strains. Therefore, it is assumed that the DXER strain has been contaminated with genes from another rat strain. For this reason, the DXER strain was excluded from further studies. The RI strains are of great interest when studying the genetics of autoimmunity, since the parental strain DA can develop induced autoimmune encephalomyelitis and induced arthritis. To get beyond the limit of resolution possible with QTL analysis the RI strains will be a powerful tool in establishing the location of quantitative trait loci affecting these disorders.

Summary of the papers
Using three different rat crosses, E3xDA, DAxDXEC and E3xLew.1F, we have been able to identify a number of genomic regions, which harbor unknown genes that partially explain the variability of the susceptibility to pristane-induced arthritis. We have identified ten such regions, Pia1 thru 9 and 11. Each of these regions has either an effect on disease onset, severity and joint erosions or chronicity. These findings demonstrate that different genes control different temporal phases in this chronic self-perpetuating disease. Still, some of the pieces in this jigsaw puzzle are missing. This is demonstrated by the inability to predict the outcome based upon the genetic composition of the animal.

We have observed that the effects from some of these autosomal loci are gender specific. E.g., the locus Pia2 on the rat chromosome 6 is associated with the onset of disease. There is a significant difference in disease onset between the group of female rats, which carries the susceptible allele and those that do not. Among male rats, this locus does not show any effect. Thus indicating that the underlying gene is dependent upon a female context. Furthermore, the disease-promoting allele is derived from the resistant strain E3. The finding that the resistant strain carries susceptibility alleles has also been observed in other loci.

Furthermore, we have suggestive evidence for the presence of genetic epistasis. Two loci (Pia1 and Pia7) identified in the cross DAxDXEC were not observed in the other crosses. However, when re-analyzing the E3xDA cross with a model for a two-locus interaction, the loci were identified and were dependent on other loci in order to have any effect.

Discussion

The animal model
Strains of rodents, which have been used in studies of various characteristics, whether in the field of physiology, behavior, pathology or genetics, have in common the fact that they have been subject to inbreeding for numerous generations, in order to isolate the desired traits. Undoubtedly, the use of inbred strains is a powerful tool to study isolated variables in the absence of
genetic variation and with control over the environmental factors. The genetic variation among the available inbred rodent strains can be considered limited due to the restricted number of founders that have been used to create them (Beck et al., 2000).

In certain strains, prone to the development of autoimmune disease, there is a tendency to identify the same loci, regardless of what disease is being studied. The strains that were used in the study on genetic predisposition to pristane-induced arthritis, were also used to study the genetic regulation of EAE (Bergsteinsdottir et al., 2000). In these studies, almost all loci associated to PIA co-localized with loci associated to EAE and to a large extent displayed an effect on the same traits. In fact, browsing through the literature on rat models for RA, shows that, out of 19 QTLs identified in five different crosses, that have in common that they utilize the DA strain as the susceptible strain, only seven do not co-localize with any of the other QTLs (see table 4).

Table 4. *QTLs identified in different crosses utilizing the rat strain DA*

<table>
<thead>
<tr>
<th>Crosses</th>
<th>DA x BN</th>
<th>DA x ACI</th>
<th>DA x F344</th>
<th>DA x LEW</th>
<th>DA x E3</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNO 1</td>
<td></td>
<td></td>
<td>Cia2</td>
<td>Pia9</td>
<td>Pia8</td>
</tr>
<tr>
<td>RNO 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNO 3</td>
<td></td>
<td></td>
<td>Cia7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNO 4</td>
<td></td>
<td></td>
<td>Cia13</td>
<td>Cia3, Aia3</td>
<td>Oia2</td>
</tr>
<tr>
<td>RNO 7</td>
<td></td>
<td></td>
<td>Cia4, Cia8</td>
<td></td>
<td>Pia2, Pia5, Pia7</td>
</tr>
<tr>
<td>RNO 10</td>
<td></td>
<td></td>
<td>Cia5</td>
<td>Oia3</td>
<td>Pia4</td>
</tr>
<tr>
<td>RNO 12</td>
<td></td>
<td></td>
<td>Cia12</td>
<td></td>
<td>Pia11</td>
</tr>
<tr>
<td>RNO 16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Co-localizing QTLs identified in the different crosses.
RNO 1 : Pia9, Cia2
RNO 4 : Pia5, Cia3, Aia3, and Pia7, Oia2, Cia13
RNO 10 : Cia5, Oia3
RNO 12 : Pia4, Cia12

The remaining twelve QTLs are distributed over five distinct chromosomal regions. These loci may harbor genes that have a common function in the disease, which makes the DA strain predisposed to both collagen and adjuvant induced arthritis. RA, for example, displays considerable clinical heterogeneity and may represent a set of distinct diseases; a syndrome that shares certain characteristics, rather than a single disease (Weyand et al., 1998). Each of these subtypes of RA may involve both specific and common pathogenic pathways that lead to disease. From this perspective, the data support the idea that each animal model may represent only a limited part of the disease or a specific subset of pathogenic events that can result in disease.
Overlapping QTLs suggests the presence of common autoimmune susceptibility genes

Over the past few years, the field of bioinformatics has evolved with tremendous speed. As a consequence of this, the scientific community has been provided with tools, available over the Internet, which has made retrieval of complex information a simple task. Such undertakings were nearly impossible a decade ago. Some of these tools involve the databases that contain information on homologies between species with regard to genes and genetic markers. This has made it possible to construct comparative genomic maps. One of the most prominent sites for mouse genome informatics is maintained by the Jackson Laboratory (www.informatics.jax.org). This site, among many other things, contains comparative genome maps between several species, including mouse, rat, and man.

In QTL analysis, these comparative maps are very interesting, since they allow for comparison of findings between the species. The illustrations below (figure 5) show the rat chromosomes where we have found loci that are associated with pristane-induced arthritis. Also, a selection of QTLs for various autoimmune diseases that are linked to these chromosomes are included. The QTLs that co-localize with Pia 1-9 and 11 are tabulated in table 5.

Table 5. QTLs identified in other studies of autoimmune diseases, in rat, and mouse that co-localizes with Pia1-9 and 11.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Chr</th>
<th>QTLs of autoimmune diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pia1</td>
<td>20</td>
<td>Defining the MHC-locus*</td>
</tr>
<tr>
<td>Pia2</td>
<td>4</td>
<td>rEae11(Bergsteinsdottir et al., 2000)</td>
</tr>
<tr>
<td>Pia3</td>
<td>6</td>
<td>rEae9(Bergsteinsdottir et al., 2000), mTnf(Libert et al., 1999)</td>
</tr>
<tr>
<td>Pia4</td>
<td>12</td>
<td>rCia2(Remmers et al., 1996), rEae5(Bergsteinsdottir et al., 2000), rEau2(Sun et al., 1999), mLbw3(Kono et al., 1994)</td>
</tr>
<tr>
<td>Pia5</td>
<td>4</td>
<td>rAia3(Kawahito et al., 1998), rCia3(Griffiths et al., 2000), rEau1(Sun et al., 1999), Scwia1(Wilder et al., 1999), mIdd20(Rogner et al., 2001), mCia3(McIndoe et al., 1999)</td>
</tr>
<tr>
<td>Pia6</td>
<td>14</td>
<td>rEae10(Bergsteinsdottir et al., 2000), mLmb2(Vidal et al., 1998), mBb3(Weis et al., 1999)</td>
</tr>
<tr>
<td>Pia7</td>
<td>4</td>
<td>rCia3(Remmers et al., 1996), rCia4(Furuya et al., 2000), rOia2(Lorentzen et al., 1998), mLbw4(Kono et al., 1994), mIdd19(Rogner et al., 2001)</td>
</tr>
<tr>
<td>Pia8</td>
<td>1</td>
<td>rCia2(Griffiths et al., 2000), mCia7(Yang et al., 1999), rEae4(Butterfield et al., 1998)</td>
</tr>
<tr>
<td>Pia9</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Pia11</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>
Table 5. continued...

All QTLs have a prefix indicating in what species it was identified.  

r = rat, m = mouse.  

* Refers to chromosome in the rat.  

* All models for autoimmune disorders listed here show association to the MHC locus.

List of abbreviations

Aia = Avridine induced arthritis  
Bb = Borrelia Burgdorferi induced arthritis  
Cia = Collagen induced arthritis  
Ciaa = Collagen induced arthritis antibody  
Eae = Experimental allergic encephalomyelitis  
Eau = Experimental autoimmune uveitis  
Idd = Insulin dependent diabetes  
Iddm = Insulin dependent diabetes mellitus  
Lmb = Lupus in MRL x C57BL/6 mice  
Lwb = Lupus white and black  
Oia = Oil induced arthritis  
Scwia = Streptococcus cell wall induced arthritis  
Sle = Systemic lupus erythematosus  
Tnfp = Tumor necrosis factor protection

The use of different animal models for induced arthritis has resulted in the identification of numerous loci associated with the disease. Some loci seem to be common between the models, while some loci are specific for a certain model. This is consistent with the observation that each model has its own set of phenotypic characteristics, while they at the same time displaying similarities to others.

There are numerous publications that claim that susceptibility loci for autoimmune diseases are clustered (Becker et al., 1998; Bergsteinsdottir et al., 2000; Cornelis et al., 1998; Furuya et al., 2000; Joe et al., 1999; Kawahito et al., 1998; Listwak et al., 1999; Martin et al., 1999; Merriman et al., 2001). A compelling hypothesis would be that certain genes are of importance for the regulation of autoimmunity, rather than in regulating a specific disease. It would be of utmost interest to clone and characterize these genes, in order to understand the development of autoimmune disorders and to identify targets for novel therapeutic methods. On the other hand, one could say that these loci coincide due to a correlation with something that occurs after the actual outbreak of autoimmunity, i.e. a secondary effect. However, an important fact remains, and that is that many of these loci are identified in studies of both rodents and humans, which suggests that there are common genetic factors that are involved, directly or indirectly, with an autoimmune response.
Figure 5. Illustration of rat chromosomes where we have identified loci associated with pristane-induced arthritis. The width of the shaded boxes show the 95% CI for the PIA loci. The solid lines indicate loci that are associated with models for autoimmune disorders in rats. The dashed lines indicate mouse loci.
Gender differences

The susceptibility to autoimmune disorders is clearly different between females and males. From an epidemiological study of 24 autoimmune diseases in the United States, it was found that the risk of becoming affected was 2.7 times greater for women compared to men (Jacobson et al., 1997). Among SLE patients, women are affected 9 times as often as men (Beeson, 1994). RA shows an overrepresentation of affected women, with a female to male ratio of 3:1 (Beeson, 1994). The same phenomenon has also been observed in animal models of autoimmune disorders (Furuya et al., 2000; Gulko et al., 1998; Holmdahl et al., 1986; Joe et al., 2000).

Different explanations for this gender bias have been proposed, including loci residing on the sex chromosomes (Ebers et al., 1996; Jawaheer et al., 2001; Santiago et al., 1998), sex hormonal effects (Holmdahl et al., 1986; Jansson et al., 1994), imprinting (Pugliese et al., 1994), and non-random X inactivation (Chitnis et al., 2000).

In both the E3xDA, and E3xLew.1f crosses, it was observed that, in the F1 population, female rats had a significantly earlier onset and a more severe arthritis. In fact, only the female F1 progeny developed disease in the cross E3xLew.1f (4/5 female, and 0/4 male rats). Analysis of the cross E3xDA with genetic markers on the X chromosome revealed no association with disease. On the other hand, we have identified three loci (Pia2, Pia5, and Pia8) that have an effect in only one of the sexes. These findings suggest a genetic basis, exerted by autosomal genes for the gender bias.

The observation that specific loci exert an effect in only one of the sexes will have implications for how the presence of predisposing genetic factors should be evaluated.

Interactions

In these studies, we have found supporting evidence for the presence of epistasis. In the cross E3xDA, we detected a suggestive epistatic effect. The use of the term ‘epistasis’ is not always the same. Here, I use the term ‘epistasis’ to describe the interactive effect occurring between two loci, where the first locus is a QTL and the second locus determines whether or not the first locus will cause a phenotypic effect.

In our case, we observed that certain alleles at a locus were a prerequisite for another locus in order to have any phenotypic effect. It is a compelling thought to imagine this required locus as an ON/OFF switch for the identified QTL. Previously, similar observations have been reported (Kuida and Beier, 2000; Lark et al., 1995; Shimomura et al., 2001). These findings need to be confirmed, preferably in double congenic strains, in which to rule out the possibility of random effects as the cause of detection.
In summary, epistatic effects could play an important role in contributing to the genetic complexity of arthritis development and progression. To fully comprehend the genetic basis for a complex trait like this, it will require an understanding of how multiple genes interact with one another to cause disease. The presence of gene interactions could also have consequences for how to evaluate the presence of predisposing genetic factors in patients.

Temporal shift of susceptibility loci
Different QTLs are identified at different temporal phases of the disease. In the PIA experiments presented here, disease development in the progeny from each cross have been followed and scored 1-2 times a week. The disease scoring at each time point was subsequently used in QTL analysis. It turned out that the effects of the severity associated loci were exerted in non-overlapping temporal intervals (see figure 6).

Figure 6. The illustration shows the timing of different pristane-induced arthritis associated loci. These loci were identified using clinical scoring at different time points as quantitative traits.

This could be explained by the occurrence of critical events in the disease progression that are driven by different sets of genes. One could argue that these effects are caused by the fact that individual rats develop disease at different time points and that we have not used disease onset as a baseline for the disease progression. I believe that this will not cause a problem in the analysis, since the majority of the rats develop disease in a similar way, with respect to time. The temporal shift has also been observed in a rat model for MS (Bergsteinsdottir et al., 2000).

The idea that these timing events could have a genetic basis could certainly be important in future experiments. E.g. in investigating differences in gene expression, it should be very interesting to specifically pay attention to
changes in expression patterns in the temporal intervals, which could mark the transition from an initial acute disease to severe erosive inflammation and eventually, the progression into a chronic phase.

Future studies
I can imagine two future approaches, both of course aimed at identifying factors that we can control and thereby, treat or prevent disease.

The first approach would directly focus on identifying and characterizing genes in the genomic regions that show association to different autoimmune diseases. A strong argument in favor of this is that several autoimmune disorders seem to be regulated by a common set of loci, which could contain genes that are of importance in the development of autoimmune disorders as a whole, rather than just for specific diseases. Today, the knowledge and the technology is available to commence with such a task. Many of the regions, which have been postulated to contain genes of importance in the development of autoimmune disorders, will need to be refined. This can be achieved by, congenic breeding or the use of advanced intercross lines. The available whole-genome sequence of man and mouse should make it possible to identify most, if not all, genes in the regions of interest. Also, gene expression profiling studies could possibly identify candidate genes in these regions. Ultimately, the effect of the identified genes must be confirmed. A possible and powerful approach to this problem could be to utilize the transgene technique, in which either individual genes or larger genomic fragments contained in artificial chromosomes, such as in BAC transgenic animals, could be tested.

An alternative future approach would therefore focus on investigating how the known disease-associated regions act together in well-defined experiments utilizing the whole span of available techniques in experimental animals. I imagine this undertaking as a jigsaw puzzle, where some pieces may be obtained from congenic strains containing different combinations of loci and other pieces obtained using transgene technique, with combinations of predisposing chromosomal regions. Ultimately, these experiments could answer questions about how and which genes act together to provide conditions under which disease can occur.
Concluding remarks

Why do we carry genes that are involved in the development of severe diseases? Are genetic disorders caused by ‘disease genes’ or by unfortunate combinations of naturally occurring polymorphisms?

Natural selection is the means by which organisms adapt to a changing environment by favoring individuals that carry gene variants that give them a reproductive advantage. Why then, have we retained variants of genes that confer predisposition to severe diseases?

There are instances where the benefit of inheriting a potential disease causing gene variant seems to be greater than the risk of developing disease. A classical example, often brought forward in this discussion, is the case of Sickle cell anemia. This is an autosomal recessive disease, caused by a point mutation in the hemoglobin beta gene (HBB). The allele frequency of HBB is significantly higher in zones with a high incidence of malaria, since this gene has a protective effect against this disease. This serves as an example of a gene that gives the carrier a reproductive advantage, while it can simultaneously cause a severe blood disorder in its homozygous form.

Other disorders could have emerged quite recently as a result of new lifestyles that have been adopted along with global industrialization. ‘Normal’ gene variants could thus suddenly start acting as disease associated genes when new environmental factors are encountered.

It has also been proposed, that genes associated with complex disorders, which are neither sufficient nor necessary to cause disease, are subject to very limited selective pressure (Pritchard, 2001).

Seemingly, there could be several alternative explanations to why we carry genes that increase the risk of developing diseases. The examples above, illustrates the complexity of many disorders where genes can be both beneficial and deleterious, interact with the environment and with other genes.

In this study, I have investigated genetic factors that confer susceptibility to rheumatoid arthritis in a rat model. This work has led to the identification of several chromosomal regions, containing uncharacterized genes that directly or indirectly are associated with the arthritis development in these rats. We have observed that timing, gender and genetic interactions are features that influence the effect that these genetic factors exert. My belief is that, intensive investigations of how the underlying genes interact with each other and the environment will be crucial for understanding the etiology and progression of the disease.
Acknowledgements

Finally I would like to express my sincere gratitude to all the people who have helped me in one way or another during my graduate studies.

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Slutligen vill jag passa på att tacka min familj och mina vänner för allt sånt som inte anknyter till studierna men som är så nödvändigt för att sträva vidare och hålla humöret uppe.

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