

Linkage of cytokine genes to rheumatoid arthritis. Evidence of genetic heterogeneity

Sally John, Anne Myerscough, Angela Marlow, Ali Hajeer, Alan Silman, William Ollier, Jane Worthington

Abstract

Objective—To investigate linkage of candidate disease susceptibility genes to rheumatoid arthritis (RA) in affected sibling pair families stratified for specific clinical features.

Method—Two hundred RA affected sibling pair families were genotyped for informative microsatellite markers mapping within or less than 3cM from: $INF\alpha$, $INF\gamma$, $INF\beta$, $IL1\alpha$, $IL1\beta$, $IL1R$, $IL2$, $IL6$, $IL5R$, $IL8R$, $BCL2$, $CD40L$, $NOS3$, $NRAMP$, α_1 anti-trypsin, and α_1 anti-chymotrypsin, using fluorescence based automated technology. Linkage was examined by defining allele sharing sibling pairs. This was assessed by maximum likelihood—inheritation by descent methods.

Results—An increase in allele sharing was seen for $IL5R$ in female sibling pairs (LOD 0.91, $p = 0.03$), for $INF\gamma$ in sibling pairs with an affected male (LOD 0.96, $p = 0.03$) and most significantly for $IL2$ in sibling pairs where one or both were persistently seronegative (LOD 1.05, $p = 0.02$).

Conclusion—Weak evidence of linkage of RA to $IL5R$, $INF\gamma$, and $IL2$ has been detected in clinical subsets of sibling pairs suggesting that RA is a genetically heterogeneous disease.

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Rheumatoid arthritis (RA) is a chronic, relapsing inflammatory condition of unknown aetiology. Twin¹ and family studies² provide evidence to support the involvement of both genetic and environmental factors in the aetiopathogenesis of RA. One measure of the size of the genetic component of susceptibility is the degree of familial clustering (λ_s),³ which is calculated from the ratio of the risk of RA in siblings of patients and the population prevalence. Estimates of λ_s for RA vary between 2–10 depending on the sibling recurrence risk and population prevalence values used in the calculation. Using a sibling recurrence risk of 3.9%¹ and a population prevalence of 0.8%² a λ_s of 4.9 is obtained. To date only HLA-DRB1 on chromosome 6 has been confidently identified as being linked to RA susceptibility. A number of alleles of this locus contain the “shared epitope”^{3,4} sequence and are associated with the disease. Analysis of the first 100 families of the ARC National Repository indicates that HLA accounts for approximately 40% (λ_{HLA} 1.8) of the total genetic component of susceptibility.⁵ Thus the majority of the genetic risk of RA is still to be accounted for, although as HLA is

the major disease locus the remaining genetic effect is likely to result from a number of further loci.

Screening of the genome for non-HLA disease susceptibility genes is currently in progress in RA using microsatellite markers for linkage analysis in large numbers of affected sibling pair (ASP) families. Simulations and experience to date suggest that detection of disease genes with a small λ_s (<1.8) will require many hundreds of families and markers at much closer intervals than the average of 11 cM currently being used. Limited results from French⁶ and British⁷ studies of 200 RA ASP families detected linkage to HLA but no reproducible effects at any other loci. An alternative approach is to apply the same microsatellite technology to sibling pair families to target likely candidate susceptibility genes. The pathology of RA indicates several molecules, which may be of potential importance in disease susceptibility. For example, many cytokines have been shown to be up regulated in the synovial tissue of RA patients⁸ and are produced spontaneously by synovial cells taken from RA patients. To identify the genes that are critical to the development of RA compared with those that are up regulated as part of the inflammatory process, it is necessary to investigate aetiology at a genetic level. Highly polymorphic markers close to gene sequences can be used in family based linkage analysis studies to directly test candidate genes. Investigating candidate genes in this way has the advantage that as the marker selected is genetically very close to the candidate gene, there is a greater chance of detecting even weak linkage.

Many of the proteins implicated in the pathology of RA will be involved as a consequence of the disease process and not a cause of it. One approach to identify molecules involved with the aetiology of RA is to identify genes that are linked to RA. Here we report on linkage analysis of 200 multi-case RA families for 16 potential candidate genes, to test the hypothesis that these genes are linked with RA. Analysis of 200 ASP families using markers within 3cM of candidate genes will permit genetic effects as small as λ_s 1.5 to be detected. In view of the clinical and genetic heterogeneity within RA we have stratified the data for; age at onset, sex, seropositive disease, and HLA haplotype sharing.

Methods

FAMILY MATERIAL

DNA was available from 200 RA families collected for the ARC National Repository for family material. Pedigrees for the first 100

Arthritis and Rheumatism Council's Epidemiology Research Unit, University of Manchester, Manchester

Correspondence to: Dr S John, ARC Epidemiology Research Unit, University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT.

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Table 1 Panel of dinucleotide repeat markers used in linkage analysis studies

Genes	Locus	Marker	Position in locus	Max Het	Reference or GDB accession number
BCL	18q21.33	BCL2	Unknown	0.57	GDB:225700
CD40L	Xq26	CD40L	3= UTR	0.9	11
IL6	7p21-p15	D7S493	2.8cM	0.89	12
IFN1A, IFN1B	9p22	IFNA	Unknown	0.72	13
IFNG	12q24.1	IFNG	Intron 1	0.67	14
IL1A, IL1B IL1RN	2q13	IL1A	Intron 5/6	0.75	GDB:157015
IL2	4q26-q27	IL2	Unknown	0.89	15
IL5R	3p26-p24	IL5RA	Unknown	0.78	15
NOS3	7q36-q24.1	NOS3	Intron 13	0.92	16
NRAMP, IL8R	2q35	VIL1	155kb	0.765	17
PI, AACT	14q13.2	PI	5'	0.9	18

Max Het: reported maximum heterozygosity of markers.

families have been published⁹ and are accessible on the ARC ERU web site (address <http://130.88.95.76/natrep.html>) together with pedigrees for the second 100 families. All family members were seen and examined according to a standard protocol. Information was also obtained from physician records and rheumatoid factor (RF) measured to classify subjects as having RA if they satisfied the 1987 ACR criteria modified for genetic studies.¹⁰ In total, 281 affected sib-pairs were included of which 101 sib-pairs were made up from families with between three and seven affected siblings. DNA from both parents was available for 41 families, and from one parent in a further 25 families. Unaffected siblings were genotyped to infer missing parental genotypes. In total 703 individuals were available for genotyping.

CANDIDATE GENE MARKERS

A set of 11 markers with reported heterozygosity ranging from 0.57–0.9, which mapped within or less than 3cM from 16 candidate genes has been compiled from published data and data held on the genome data base (GDB) (table 1). The candidate genes include a number of cytokines, interferon α (IFN α), interferon β , the interferon γ gene cluster, interleukin 1 α , (IL1 α), interleukin 1 β , interleukin 1 receptor, interleukin 2 (IL2), interleukin 6 (IL6), interleukin 5 receptor (IL5R), and the interleukin 8 receptor (IL8R). The other candidate genes are B-cell lymphoma 2 (BCL2), CD40 ligand (CD40L), natural resistance associated macrophage protein (NRAMP1), endothelial nitric oxide synthase, (NOS3) α_1 anti-trypsin (PI) and α_1 antichymotrypsin (AACT).

MICROSATELLITE GENOTYPING

Semi-automated analysis of microsatellite genotypes was performed using a PE Applied Biosystems 377 DNA sequencer with Genescan analysis and Genotyper software (PE Applied Biosystems). Forward polymerase chain reaction (PCR) primers were fluorescently labelled with either 6-FAM, HEX or TET attached to the 5' end of the primer during synthesis. Some of the published primer sequences were redesigned to produce products of different sizes that could be incorporated into the panel. Table 2 gives details of the primers. PCRs were performed in a total reaction volume of 10 μ l, containing 50 ng of DNA,

10 pmol of each PCR primer, 4 nmol of each of the four deoxynucleotide triphosphates, 0.2 units Taq polymerase (Biolone) in 1–3 mM MgCl₂ buffer (table 2). The mixture was overlaid with a drop of liquid paraffin. The PCRs were performed in 96 well microtitre plates on Hybaid Omnigene thermal cyclers with 30 cycles of denaturation (one minute, 95°C), primer annealing (one minute, at the annealing temperature indicated in table 2) and extension (45 seconds at 72°C). Reactions for each marker were performed separately. PCR products were combined into a single pool before electrophoresis, such that all 13 PCR products for a person could be loaded onto a single lane of a gel. Electrophoresis was performed on 0.2 mm 4% polyacrylamide gels run for two hours at 3000 V with a running temperature of 51°C. Allele sizes were expressed as mobility units (approximately equivalent to base pairs) as measured by GENESCAN 1.2 analysis software, using TAMRA 350 size standards (PE Applied Biosystems). PCR products from two DNA reference samples were included on every gel as a control to monitor any gel to gel variation.

DATA ANALYSIS

Sib-pair analysis methods were used to test for excess allele sharing between sibs at all of the loci tested. Sharing at each locus was quantified by the number of alleles shared identical by descent (IBD), alleles shared IBD have the same genotype and the same ancestral origin. Under the null hypothesis of no linkage IBD sharing of 2, 1 or zero alleles is expected to be in the proportion 1:2:1, an increase in the proportion sharing two alleles indicates linkage. As RA has a comparatively late age at disease onset, parental data are often missing, making it difficult to assign IBD sharing directly. The maximum likelihood score (MLS) method was therefore used to estimate allele sharing. The MLS IBD method,^{19–21} allows for uncertainty of IBD assignment resulting from unknown marker haplotypes or missing parental genotype data by inferring most likely IBD sharing probabilities. MLS IBD was carried out in the SPLINK package, version 1.05, (David Clayton MRC Biostatistics unit, Cambridge). In this analysis the IBD data from multiple affected sibships were given the conservative weighting of 2/n where n is the number of affected sibs.

The transmission disequilibrium test²² (TDT) is a test of linkage in the presence of an association. Parents heterozygous for a marker are used to test for deviation from random 50:50 transmission from the parent to an affected offspring for one or more alleles of a candidate marker. A maximum likelihood method of TDT was carried out in TRANSMIT, version 2.1 (David Clayton, MRC Biostatistics unit Cambridge). In the TDT, the proband from each pedigree was used in the analysis.

Stratification of the data

Data were analysed as a complete set and for certain markers where some deviation from

Table 2 Primer sequences, fluorescent label, and expected size range of PCR products in base pairs (bp) and PCR conditions for the markers in the candidate gene panel

Marker	Label	Product size bp	Annealing temperature (°C)	MgCl ₂ concentration (mM)	Primer sequences
IL5R	FAM	83–105	50	1.5	AATGATCTTTTCTAGGTAGA CCTCTGGAGCTTGAGATA
IL2	FAM	113–149	55	2	AAAGAGACCTGCTAACAC CCTATGTTGGAGATGTTTAT
CD40L	FAM	229–261	50	1	AAGAAGAGAACTGACTAGCAACG TCGGACAGTTATTTCATTCTTT
IFN γ	HEX	122–136	40	1.5	AAAAGATAGTTCCAAAC TTATAATTATAGCTGTC
D7S493	HEX	193–229	55	1.5	GGAAGTCCCAGCCATAGTT GAAAGCACTTACCTACTGAGGATTT
IFN α	HEX	229–251	62	1.5	CCAGGGCCTTGTCTGTGTGGA* AAGGTGGAACCCCACTGGA
NOS3	HEX	263–319	57	1	CCCCCGGAGAAATGGAGAGGTG* TGGAAATGACTTGGGGACGCG
D2S1471	TET	81–113	55	1.5	TACCATTACACATCGAAGAAC GATCTCTCCCCACAATAAC
IL1 α	TET	131–145	55	1.5	ATGTATAGAATTCATTCTCTG TAAAATCAAGTGTGTGATGTAG
PI	TET	155–189	55	1.5	GCATCACACAGAGACACGGAT TTGCAGGGAGTCAGGTGTATG
BCL2	TET	267–285	62	1.5	CGGCTAGAAAGGTTCCAGGCG* ACTTGGTGGTCGCTGGGGTCC

*Primer sequences have been redesigned from published sequences.

random inheritance was seen, a number of stratifications were made to the data set as specific genetic effects may be greater in some subgroups of patients.^{23–25} The following criteria were used for stratification:

(1) Age at onset—both siblings having an age of onset of less than 50 years were compared with those where one or both siblings had a later age of onset. It is predicted that stronger genetic effects would be seen in siblings with a younger age at onset.

(2) Sex—As there is evidence that male RA is genetically different, female/male and male/male sibling pairs were compared with female/female pairs. There were insufficient male/male pairs for direct comparison.

(3) Disease severity—sibling pairs who both have erosions and who were “ever seropositive” were compared with sibling pairs with a less severe disease. The less severe disease group contained 49 sibling pairs with at least one sibling who had no erosions and 47 sibling pairs with at least one sibling who was “persistently seronegative”. There were eight sibling pairs who were both persistently seronegative and only four sibling pairs who both had no erosions.

(4) HLA haplotype sharing—as HLA is known to be the major susceptibility locus in RA, sib-pairs were subdivided into two groups

by IBD status at HLA. One group of families showing stronger evidence of linkage to HLA was compared with those discordant for HLA background—that is, sharing 1 or zero HLA haplotypes. It would also be interesting to compare siblings who were “shared epitope” positive with those who were “shared epitope” negative, however the numbers of “shared epitope” negative pairs (14) was too small to analyse.

Results

WHOLE DATA SET

Estimated IBD sharing generated by SPLINK did not generate any significant LOD scores for any of the markers tested (table 3) although a small increase in sharing of two alleles IBD was seen for markers IL2, IL5R, and IFN γ .

STRATIFICATION OF THE DATA SET

RA is an extremely heterogeneous condition, and as it is expected that different genes may contribute to variation in disease expression, the whole data set was stratified as described in the methods. MLS IBD analysis was performed on the subgroups for markers IL5R, IL2, and IFN γ , where some deviation from random sharing had been seen in the whole data set. No significant deviation from random allele sharing was seen in the sibling pairs stratified by age at onset of RA (table 4 (A)).

Two LOD scores were significant at $p < 0.05$ level in the patient group stratified by sex (table 4(B)). IL5R showed an increase in two alleles sharing in the all female pairs (LOD 0.9, $p = 0.03$) and a similar increase was detected for IFN γ in pairs with a male—that is, male/male and male/female pedigrees (LOD 0.9, $p = 0.03$).

The data set was also subdivided to test for linkage in sibling pairs with more severe disease. The strongest evidence of linkage was for IL2 in the group with less severe disease (LOD 1.05, $p = 0.02$ (table 4 (C))). Many patients in this group were seronegative and to

Table 3 MLS IBD analysis using SPLINK

Marker	Het	Total IBD assigned 0 : 1 : 2	χ^2	Equivalent LOD
IFN α	0.68	59 : 118 : 61	0.05	0
IFN γ	0.63	57 : 107 : 74	1.8	0.39
IL1 α	0.78	49 : 106 : 49	0	0
IL2	0.89	37 : 102 : 53	1.73	0.38
IL5R	0.79	48 : 89 : 60	1.84	0.39
D7S493 (IL6)	0.87	50 : 105 : 51	0.02	0
BCL2	0.36	56 : 100 : 55	0.12	0.03
D2S1471 (NRAMP,IL8R)	0.79	29 : 54 : 22	0	0
NOS3	0.91	50 : 75 : 47	0.25	0.05
PI	0.85	55 : 99 : 58	0.49	0.1
*CD40L	0.75	50 : 56	0	0

Estimated heterozygosity (Het) is calculated from parental allele frequencies. Equivalent LOD: χ^2 can be converted to a LOD score by $\chi^2/4.86$. *CD40L is on the X chromosome, therefore 1:1 allele sharing is shown from the maternal chromosome, under random inheritance this is 50:50.

Table 4 MLS IBD analysis in patient subgroups for IFN γ , IL5R, and IL2

Marker	Patient group	Total IBD assignment 0 : 1 : 2	χ^2	Equivalent LOD	p Value
(A)					
IFN γ	Early onset	29 : 67 : 37	0.48	0.1	NS
	Late onset	24 : 39 : 38	2.59	0.56	0.08
IL5R	Early onset	33 : 59 : 40	0.97	0.2	NS
	Late onset	21 : 43 : 28	0.74	0.16	NS
IL2	Early onset	17 : 59 : 32	2.8	0.6	0.07
	Late onset	20 : 42 : 19	0	0	NS
(B)					
IFN γ	All female	33 : 64 : 31	0	0	NS
	female/male, male/male	24 : 43 : 42	4.43	0.96	0.03
IL5R	All female	26 : 40 : 38	4.20	0.91	0.03
	female/male male/male	23 : 49 : 23	0	0	NS
IL2	All female	23 : 52 : 26	0.07	0	NS
	female/male, male/male	13 : 51 : 27	2.50	0.54	0.08
(C)					
IFN γ	Severe disease	21 : 36 : 22	0.16	0.04	NS
		35 : 72 : 51	1.7	0.38	NS
IL5R	Severe disease	21 : 34 : 21	1.02	0.2	NS
		31 : 62 : 39	0.8	0.19	NS
IL2	Severe disease	16 : 38 : 13	0	0	NS
		19 : 64 : 41	4.85	1.05	0.02
(D)					
IFN γ	HLA concordant	17 : 33 : 23	0.76	0.18	NS
	HLA discordant	32 : 52 : 38	0.81	0.18	NS
IL5R	HLA concordant	16 : 30 : 20	0.48	0.1	NS
	HLA discordant	24 : 41 : 30	1.0	0.24	NS
IL2	HLA concordant	12 : 35 : 14	0.1	0.02	NS
	HLA discordant	20 : 50 : 29	1.16	0.25	NS

test if IL2 is linked specifically to seronegative RA we analysed sibling pairs where one or both were seronegative. Despite the smaller sample size of this subgroup, the LOD score was maintained at 1.07 ($p = 0.02$) with final total IBD sharing assignments of (0-IBD: 1-IBD: 2-IBD) 9:35:25. No significant deviation from random allele sharing was seen in the families stratified by HLA sharing (table 4(D)). The marker for NRAMP (D2S1471) was also analysed in the HLA subgroups as some weak evidence of linkage had previously been shown in a smaller set of ARC families²⁶ and in patients discordant for HLA haplotypes.²⁷ In this larger data set, no deviation from random sharing was observed (data not shown).

TDT ANALYSIS

As IFN γ , IL5R, and IL2 are all markers within the gene sequence, evidence of linkage to these genes would be further supported by demonstration of an association between specific alleles of the microsatellite marker and RA, result-

Table 5 TDT analysis of 16 alleles of the IL2 microsatellite in the erosion and/or RF negative subset of patients

Allele size (bp)	Observed transmission to offspring	Expected transmission to offspring	
113	28	27	
119	1	1	
121	3	4	
123	6	5	
125	8	8	
127	49	58	
129	22	22	
131	63	62	
133	34	33	
135	73	73	
137	32	38	
139	36	36	
141	31	25	141/143 χ^2 5.3 p=0.05
143	14	8	
145	2	2	
147	2	1	

ing from linkage disequilibrium with a potential functional polymorphism in the gene. Markers for IL5R, IFN γ , and IL2 were therefore analysed by TDT in the subsets of patients where there was some preliminary evidence of linkage ($p \leq 0.05$). No evidence of linkage to a particular allele of IL5R or IFN γ was seen (data not shown). There were 16 alleles observed for the IL2 microsatellite. Table 5 shows TDT analysis of observed and expected transmission of each of these alleles. Two alleles of the IL2 marker that were only 2bp apart (141 and 143bp) appeared to be inherited by affected offspring more often than expected. If the transmission of these two alleles were combined, a χ^2 of 4.37 ($p=0.05$ one degree of freedom) was observed.

Discussion

We have investigated the contribution of a number of candidate gene loci to RA susceptibility using linkage analysis methods. Analysis of the data before stratification showed no significant deviation from expected allele sharing for any of the markers tested. Although the results were negative and a major contribution for these genes can be excluded, the actual effect of each locus that can be excluded in this data set varies depending upon a number of factors. Over 200 sibling pairs have been genotyped in this investigation, but because the markers were not fully informative and also because of an absence of parental information, the amount of information contributed by the families is variable. It is possible to exclude a genetic effect ($\lambda_s = 1.5$) for the following genes; IFN α , IL2, IL5R, NRAMP, IL8R, NOS3, and PI. For IFN γ , IL1 α , and CD40L, which were less informative, a genetic effect with a $\lambda_s = 1.7$ can be excluded. The heterozygosity of BCL2 (0.36), calculated from our data set, was much lower than the reported heterozygosity, therefore, it is only possible to exclude a $\lambda_s = 1.85$. We conclude that none of the genes tested in this investigation has a role as great as HLA, which was calculated to have a λ_s of 1.8, based on analysis of the first 100 families in the ARC National Repository.⁵

A possible reason for failing to detect weak genetic effects in complex disease is heterogeneity. To deal with this, the data were subdivided into a number of more homogeneous subsets and analysis was repeated for IL5R, IFN γ , and IL2, where some deviation from random allele sharing was observed. For each of these genes, an increase in allele sharing ($p < 0.05$) was observed in a patient subgroup.

One way of supporting the initial finding of linkage is to demonstrate linkage disequilibrium between the marker and a potential functional polymorphism within the gene. TDT detects linkage in the presence of an association. There was some evidence that the larger alleles of the IL2 microsatellite were being transmitted to affected offspring in the patient subgroup with less severe disease, more often than expected. This provides some preliminary evidence that alleles of the IL2 microsatellite may be in linkage disequilibrium with a functional polymorphism in the gene. The

evidence for linkage was seen most convincingly in seronegative RA. HLA associations have already suggested that patients with seronegative RA may represent a disease with a different genetic background.²⁸ IL2 is primarily a Th1 cytokine produced by both naive and activated CD4+ve cells, and functional polymorphisms within regulatory or coding sequences could lead to changed immune function. Interestingly, IL2 knockout mice develop severe autoimmune disease and lymphocyte proliferation,²⁹ suggesting functions of IL2 are more diverse than previously realised.

The TDT analysis did not support an association with both IFN γ or IL5R alleles and RA; there are a number of explanations. Highly polymorphic microsatellites with many alleles are not always in linkage disequilibrium with less polymorphic markers, for example, functional biallelic point mutations, which are genetically very close.³⁰⁻³¹ Linkage also extends much further than linkage disequilibrium and the linkage finding may be true but result from linkage, not with the candidate gene, but with a different gene in the same region. It is also possible that these results may be false positives. In the initial analysis on 200 families, no correction for multiple testing was made, as there was an a priori hypothesis for investigating each of the candidate genes. However, for each marker tested in the patient subsets, nine separate analysis tests were performed. It is possible that for the three genes tested in this way that 1–2 false positive results would be seen at a 5% significance level. Replication of these results in further data sets or the demonstration of an association between RA and a functional polymorphism of the genes is required to confirm which, if any of these findings are because of genuine linkage. This study has identified three genes worthy of further study and clearly illustrates the importance of considering genetic heterogeneity when investigating a complex disease such as RA.

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