Polymorphisms in the Mannose Binding Lectin (*MBL*) Gene Are Not Associated with Radiographic Erosions in Rheumatoid or Inflammatory Polyarthritis

ANNE BARTON, HAZEL PLATT, FIONA SALWAY, DEBORAH SYMMONS, MARK LUNT, JANE WORTHINGTON, and ALAN SILMAN

ABSTRACT. ***Objective.*** To investigate the association between the mannose binding lectin gene (*MBL*) promoter and structural single nucleotide polymorphisms (SNP) with development of erosions in a primary care inception cohort of patients with inflammatory polyarthritis (IP).

Methods. DNA was available from 438 patients with IP and radiographic data were available for all patients at 5 years. Four SNP [*MBL*-550*C/G (H/L), *MBL*-221*G/C (Y/X), *MBL* codon 52*C/T, and *MBL* codon 54*G/A] mapping to the *MBL* gene were genotyped using primer extension techniques. Allele frequencies were compared between IP cases with erosions by 5 years and those without.

Results. None of the SNP were associated with erosive outcomes by 5 years. Furthermore there was no association with Larsen score by 1 or 5 years or with the change in Larsen score between 1 and 5 years. Similarly, the genotype combinations known to encode for low MBL protein production were not associated with erosive outcome in the IP cohort as a whole or in those with rheumatoid arthritis (RA) by 5 years.

Conclusion. Polymorphism within the *MBL* gene is not associated with presence or extent of erosions by 5 years in patients with RA or IP. (J Rheumatol 2004;31:442–7)

Key Indexing Terms:

MBL RHEUMATOID ARTHRITIS GENETICS
INFLAMMATORY ARTHRITIS EROSIONS

Twin studies suggest a substantial genetic contribution to rheumatoid arthritis (RA) susceptibility and family studies have shown that first-degree relatives of patients with RA are at increased risk[1]. However, the increased risk in probands recruited from a community based setting is modest compared with hospital-recruited patients[2]. As hospital-based patients are likely to have more severe disease, this suggests that genetic factors may play a greater role in determining disease severity and outcome.

Mannose binding lectin (MBL) is involved in the innate immune system and it has been suggested that impairment of innate immunity as a result of MBL deficiency may lead to increased presentation of antigens to the host, a reduction in self-tolerance, and susceptibility to autoimmunity[3]. There are a number of single nucleotide polymorphisms (SNP) of the MBL gene (*MBL*) and haplotypes have been defined, some of which encode high and others low levels of MBL protein production. The wild type *MBL* allele is referred to as A. Structural polymorphisms exist at 3 positions: in codon 52*C/T, codon 54*G/A and codon 57*G/A, and the presence of such a variant allele at any of these sites is referred to as O. MBL protein levels are higher in individuals with an A/A than A/O genotype and MBL levels are virtually undetectable in individuals with O/O genotype. In addition, 2 promoter variants exist: the *MBL*-550*C/G (H/L) SNP, which has little effect on levels, and the *MBL*-221*G/C (Y/X) SNP, which has a profound lowering effect when 2 copies are present.

Studies have reported association of *MBL* polymorphisms with susceptibility to RA but the results have been conflicting[4-11]. The variation in these reports may result from differences in the patient cohorts investigated, particularly if *MBL* polymorphisms are more important in determining disease severity rather than susceptibility. Several studies have therefore attempted to address this issue and have reported association of *MBL* polymorphisms, including haplotypes known to encode low MBL protein production, with development of erosions in RA[6,7,9,10]. Furthermore, studies have also investigated MBL protein levels with outcome in RA[6,7,9,11,12], including 2 prospective studies of RA patients in which low MBL protein levels were associated with development of erosions[9,12].

From the Arthritis and Rheumatism Campaign Epidemiology Unit and the Centre for Integrated Genominc Medical Research, University of Manchester, Manchester, UK.

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A. Barton, MRCP, Clinical Lecturer; H. Platt, BSc, Experimental Officer; F. Salway, MSc, Experimental Officer; D. Symmons, FRCP, Professor of Rheumatology and Musculoskeletal Epidemiology; M. Lunt, PhD, Research Fellow; J. Worthington, PhD, Reader in Genetic Epidemiology, A. Silman, FRCP, Professor of Rheumatic Disease Epidemiology.

Address reprint requests to Dr. A Barton, ARC-EU, Stopford Building, University of Manchester, UK. E-mail: ABarton@fs1.ser.man.ac.uk

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If polymorphisms within the *MBL* predispose to the development of severe disease, then this is best determined by studying a large inception cohort of patients followed prospectively with outcome measures recorded at defined time points in the disease course. By contrast, some of the reports investigating association of *MBL* polymorphism with outcome were cross-sectional in nature[7,8,11] while previous longitudinal studies have generally had relatively small sample sizes[6,9,10] and most have investigated prevalent RA cases[6-9,11]. One recent study found that the relative risk of developing erosions in the presence of MBL insufficiency may be higher in RA patients with short disease duration[13].

Thus, the ability to detect the effect of MBL on erosive outcome may be enhanced by prospectively following an inception cohort of patients[13]. We therefore first investigated whether *MBL* genotypes known to encode low MBL protein levels predispose to the development of erosions and then determined whether these genotypes are associated with extent or progression of erosions. We studied a population-based inception cohort of subjects with inflammatory polyarthritis (IP) from the Norfolk Arthritis Register who were followed prospectively. Erosions were chosen as the outcome of interest because the development of erosions is accepted as an objective and reliable outcome measure of the disease process[14]. In addition, the presence of erosions is used in many studies as a surrogate marker for quantifying disease severity[15] and furthermore, previous studies have identified association between *MBL* SNP and erosions[6,7,10]. Finally, the identification of genetic variants that predict erosive outcome would be useful in the clinical setting to target therapies appropriately.

MATERIALS AND METHODS

Study design. A prospective cohort study was performed to investigate whether the presence of promoter and structural polymorphisms of the *MBL* played a role in determining erosive outcome in a large inception cohort of patients with IP (of which RA is a major subset). Association of 2 promoter and 2 exonic *MBL* SNP with the presence, extent, and progression of radiological erosions by 5 years was assessed.

Patients. DNA samples were available from 438 patients from a primary care inception cohort of IP recruited from the Norfolk Arthritis Register (NOAR). Details of the case ascertainment procedure have been described in detail[16]. Briefly, all cases with IP (defined as swelling of 2 or more joints lasting for 4 or more weeks) within the region formerly known as the Norwich Health Authority are notified and assessed by a research nurse using a standard questionnaire and examination. Baseline clinical data are recorded and blood taken for rheumatoid factor (RF) measurement and DNA extraction. Patients are reviewed annually and scored at each assessment as to whether American College of Rheumatology classification criteria for RA are satisfied[17]. Patients with a diagnosis other than RA, psoriatic arthritis, and post-viral arthritis are excluded from analysis.

All subjects had hand and feet radiographs at 5 years and a subset also had radiographs at 1 year as described[18] (Figure 1). Radiographs of the hands and feet are scored by 2 observers using the Larsen method[19]. A third observer arbitrated in cases of disagreement. The cases investigated in the current study were recruited between 1990 and 1994 and all had been followed up for at least 5 years. All had radiographs of the hands and feet at 5 years available for analysis.

Genotyping. Four SNP within *MBL* [*MBL*-550*C/G (H/L), *MBL*-221*G/C (Y/X), *MBL* codon 52*C/T, and *MBL* codon 54*G/A] were genotyped using primer extension techniques (SNaPshot, ABI, Warrington, UK). Two polymerase chain reaction (PCR) products were designed containing the 2 promoter or 2 exonic SNP (Table 1). For each SNP, genotyping was performed by single base extension of a forward probe and validated on a subset of the total sample using the reverse probe (Table 1). The codon 57*G/A SNP was not tested as the reported frequency in the Caucasian population was too low (5%) for useful analysis[3].

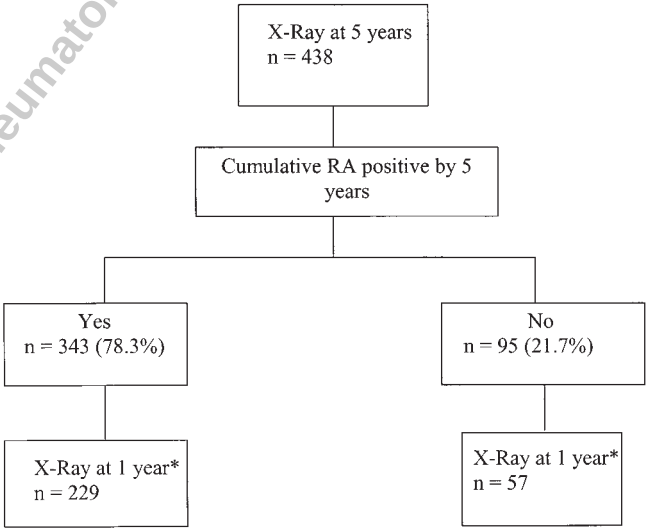

Figure 1. Description of patients with IP. *Only a subset had radiographs done at 1 year[18].

Table 1. Details of PCR primers and probes used to genotype *MBL* SNP. Genotyping was performed using the forward probe for primer extension, and genotypes were validated using the reverse probe.

MBL SNP	PCR Primers Forward, Reverse	Primer Extension Probes Forward, Reverse
MBL-550* C/G (H/L)	CCAGGGCCAACGTAGTAAGAGAGGGGTTCATCTGTGCCTA	GCTTACCCAGGCAAGCCTGTTGCTTCCCCTTGGTGTTTTA
MBL-221* G/C (Y/X)	As above	ACGGTCCCATTTGTTCTCACTGCCACTGCTGGAAGACTATA AACATGCTTTC
MBL codon 52* C/T	TGGCAGCGTCTTACTCAGAACAGGCAGTTTCCTCTGGAAG	CAGGCATCAACGGCTTCCCAGGCAAAGATGGGTGGTTCCCC CTTTTCTCCCTTGGTGCCATCAC
MBL codon 54* G/A	As above	AGGCATCAACGGCTTCCCAGGCAAAGATGGGCGTGATGCC CAACACGTACCTGGTTCCCCCTTTTCTCCCTTGGTG

Table 2. Analysis of erosive outcome (erosions yes/no) at 5 years by *MBL* SNP allele and MBL-producer haplotype. Genotyping was not successful for all samples for all assays. Hence, the total number of patient samples available for genotyping is shown at the top of the table whereas the actual number of successful genotypes is the sum of the alleles for each SNP divided by 2 or the sum of the values for the haplotypes.

SNP Allele/Haplotype	Erosive n = 194 (%)	Non-erosive n = 244 (%)	OR (95% CI)
–550			
G (H)	249 (67.6)	292 (63.5)	1.0 (referent)
C (L)	119 (32.3)	168 (36.5)	0.83 (0.62–1.12)
–221			
G (Y)	289 (77.3)	363 (79.0)	1.0 (referent)
C (X)	85 (22.7)	99 (21.0)	1.07 (0.78–1.50)
Cd52			
C (A)	333 (92.5)	414 (90.4)	1.0 (referent)
T (D)	27 (7.5)	44 (9.6)	0.76 (0.46–1.26)
Cd54			
G (A)	299 (82.6)	405 (86.9)	1.0 (referent)
A (B)	63 (17.4)	61 (13.1)	1.40 (0.96–2.05)
MBL-producer group			
1	100 (55.6)	138 (60.3)	1.0 (referent)
2	70 (38.9)	77 (33.6)	1.26 (0.81–1.96)
3	10 (5.5)	14 (6.1)	0.69 (0.28–1.69)
			p = 0.88

OR: odds ratio; 95% CI: 95% confidence interval.

Statistical analysis. Genotypes were divided into 3 groups according to whether they encoded high (Group 1), intermediate (Group 2), or low (Group 3) levels of MBL protein production. These were defined as follows: Group 1: 2 normal structural alleles with either high or low expression promoter variants at the *MBL*-550*C/G (H/L) and *MBL*-221*G/C (Y/X) positions; Group 2: one variant structural allele and one normal structural allele combined with either 2 high or one high and one low expression promoter variants at the *MBL*-221*G/C (Y/X) position regardless of *MBL*-550*C/G (H/L) genotype; and Group 3: 2 structurally variant alleles or one variant structural allele combined with homozygosity for low expression promoter variant at the *MBL*-221*G/C (Y/X) position. Allele frequencies of the 4 SNP and *MBL*-producer haplotype frequencies were compared between patients with IP who had developed erosions by 5 years with those who had not. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated. Association with presence of erosions by 5 years was also tested in the subgroups of IP patients who satisfied ACR criteria for RA by 5 years or were RF positive by 5 years.

Larsen score was used as a measure of radiographic severity. The median Larsen score and interquartile range (IQR) were calculated at 1 and 5 years by *MBL*-producer status for the IP group as a whole and in subgroups (i) satisfying and (ii) not satisfying ACR classification criteria for RA by 5 years. This was repeated restricting the analysis to cases with erosions. The 2-sample (Mann-Whitney) test was used to compare Larsen score at 1 and 5 years as well as the change in Larsen score for the *MBL* polymorphisms tested.

As the Larsen score is a count, negative binomial regression was used to model the influence of *MBL*-producer status groups on Larsen score at 5 years. Negative binomial regression was used rather than Poisson regression as the scores obtained display greater variation than a Poisson distribution. Hence, Poisson regression underestimates the standard errors. The outcome from the negative binomial regression is a multiplier for each group: for example, a coefficient of 1.2 implies that the Larsen score in that group is, on average, 20% higher than the referent group.

Finally, as treatment with disease modifying antirheumatic drugs (DMARD) may mask genetic associations, all analyses were adjusted for ever DMARD use.

RESULTS

Patients. The mean age of the IP cohort was 55.4 years (± 14.4). Sixty-five percent of patients were female (n = 298). One hundred and seventy-six patients (40.5%) had zero copies, 199 patients (45.7%) had one copy, and 60 patients (13.8%) had 2 copies of the shared epitope, a group of HLA DRB1 alleles associated with RA and sharing amino acid homology in the third hypervariable region. Three hundred and forty-three (74.9%) of the IP cohort satisfied ACR criteria for RA by 5 years. Of these, the mean age at disease onset was 53.6 years (± 14.4) and 39.9% were RF positive by 5 years. A subset of patients (n = 289) also had radiographic data at 1 year after presentation of whom 97 had erosive changes (Figure 1). Of the total 438 IP cases with radiographic data at 5 years, 194 were erosive with Larsen scores ranging from 2 to 138.

Polymorphisms. None of the SNP tested individually were associated with development of erosions by 5 years when analyzed either by allele (Table 2) or genotype. Similarly, no association by MBL protein producer status was detected with presence of erosions by 1 or 5 years (Table 2) or with RF positivity by 5 years either for the IP cohort as a whole or when analysis was restricted to cases fulfilling ACR classification criteria for RA by 5 years (data not shown). No association was detected when analysis was restricted to patients with either zero copies (p = 0.47) or 1/2 copies (p = 0.31) of shared epitope alleles.

There were no differences (Mann-Whitney test) in the median Larsen scores by MBL-producer status at either 1 (p = 0.17) or at 5 years (p = 0.12) (Tables 3 and 4, respec-

Table 3. Influence of MBL-producer status on Larsen score at 1 year: stratified by RA status.

	RA Status by 5 Years		
	RA Median (IQR; n)	Not RA Median (IQR; n)	All Median (IQR; n)
MBL-producer group			
1 (normal)	3 (0–10; n = 120)	1 (0–8; n = 31)	3 (0–10; n = 151)
2 (intermediate)	4 (0–11; n = 82)	1 (0–14; n = 20)	3.5 (0–11; n = 102)
3 (low/absent)	2 (0–7; n = 13)	0 (0–4; n = 3)	1.5 (0–6.5; n = 16)
Comparison (Mann-Whitney)*	p = 0.43	p = 0.19	p = 0.17

* Use of DMARD by 5 years included as a covariate to adjust for possible confounding by treatment and global p value presented. IQR: interquartile range.

Table 4. Influence of MBL-producer status on Larsen score at 5 years: stratified by RA status.

	RA Status by 5 Years		
	RA Median (IQR; n)	Not RA Median (IQR; n)	All Median (IQR; n)
MBL-producer group			
1 (normal)	6 (0–24; n = 175)	3 (0–14; n = 62)	5 (0–20; n = 238)
2 (intermediate)	10 (1–30; n = 111)	3.5 (0–16.5; n = 36)	5 (1–22; n = 147)
3 (low/absent)	8 (2–15; n = 19)	4 (0–6; n = 5)	6 (1.5–14; n = 24)
Comparison (Mann-Whitney)*	p = 0.28	p = 0.30	p = 0.12

* Use of DMARD by 5 years included as a covariate to adjust for possible confounding by treatment and global p value presented. IQR: interquartile range.

tively) or with change in Larsen score between 1 and 5 years (p = 0.29). Because of the very weak trend towards an increased effect on Larsen score as time progresses (p = 0.17 at 1 year and p = 0.12 at 5 years), analysis was also performed to compare Group 1 (normal MBL production) (n = 249) versus Group 3 (low/absent MBL production) (n = 24) individuals. Again, there was a very weak trend for MBL producer status to be more associated with Larsen score with increasing disease duration (p = 0.12 for Larsen score at 1 year and p = 0.10 for Larsen score at 5 years). To investigate this further, we divided the individuals into high (A/A and A/O high promoter) and low (O/O and A/O low promoter) MBL producer groups and Larsen score was again analyzed at 1 and 5 years. However, no trend was found (Larsen score at 1 year, p = 0.29; Larsen score at 5 years p = 0.35). Indeed, the median Larsen score was generally lower in the low MBL group (Group 3) than the intermediate (Group 2) MBL producer subset, but this may have been due to the small numbers of individuals in Group 3 (Tables 3 and 4). The Larsen score at 5 years was higher in individuals with intermediate MBL-producer status (Group 2) compared to the normal MBL producer status (Group 1), but lower in those with a low-producer status (Group 3) (Table 5). This was true whether patients satisfied ACR classification criteria for RA by 5 years or not (Table 5). Stratifying the data by the presence of RF did not affect the conclusions (data not shown). Although patients with low/absent MBL producer status (Group 3) had higher median Larsen scores (whether or not they could be classified as having RA) than those in Group 1, the distributions were such that the 75th percentile was lower (Table 4) as, indeed, was the mean. When the data were analyzed using negative binomial regression adjusting for DMARD use by 5 years, overall patients in Group 3 had Larsen scores at 5 years that were 35% of those in the referent group (Group 1) (Table 5).

DISCUSSION

In this large prospective study examining *MBL* polymorphisms with erosions in patients with IP, no association with promoter or structural variants or with combinations of these variants known to encode low MBL production was detected.

A prospective investigation of Icelandic patients with RA identified low MBL protein levels as being associated with development of erosions, poor treatment response, and IgM or IgA RF positivity[9], while a prospective study of Danish patients with RA showed that low MBL levels were associated with a younger age at onset of RA, a higher level of disease activity markers, and a higher annual increase in the radiographic destruction score[12]. Thus, one of the limitations of our current study may be that we have no information available as to MBL protein levels in the IP patients investigated. However, our aim was not to address whether low MBL levels predispose to severe disease but whether polymorphism within the *MBL* gene itself is responsible for

Table 5. Results of negative binomial regression for Larsen score at 5 years.

	Coefficient (95% CI) of Larsen Score at 5 Years	
	RA by 5 Years	Non RA by 5 Years
MBL-producer group		
1 (normal)	1.0 (referent)	1.0 (referent)
2 (intermediate)	1.11 (0.57–2.15)	1.17 (0.81–1.69)
3 (low/absent)	0.35 (0.08–1.60)	0.75 (0.36–1.57)

this. Neither the polymorphisms individually, nor combinations known to encode high (Group 1), medium (Group 2), or low (Group 3) MBL production were associated with presence, extent, or progression of erosions or with presence of RF by 5 years.

There are a number of methodological issues that must be considered when interpreting our results. Previous studies have investigated association of *MBL* polymorphisms in patients with RA and it may be argued that our failure to detect an association is due to the fact that analysis was not restricted to ACR-defined RA cases with established disease. However, first, the majority (75%) of the IP cases did satisfy ACR criteria for RA by 5 years. Second, neither stratifying the data for presence of RF nor restricting analysis to those who fulfilled ACR criteria for RA by 5 years affected the conclusions. Finally, as erosions are part of the criteria for RA, we have investigated association in an inception cohort of patients all followed prospectively unselected for the presence of erosions at study entry. This study design should eliminate any bias that might arise from studying RA prevalent cases with variable disease duration. For example, in one previous large study, *MBL* promoter and structural alleles were reported to be associated with development of erosions, but the RA patient group with erosive disease had a significantly longer disease duration than patients with non-erosive disease[7].

The effects of treatment on disease severity may obscure the investigation of genetic factors. Patients with severe disease at baseline who receive treatment early are expected to have milder disease over time than would be expected if they remained untreated. We attempted to account for this possible confounding by treatment in the analysis, but the adjustment applied (whether patients had ever received a DMARD) was rather crude and may not have eliminated the effect completely.

The negative results are unlikely to have arisen due to a type 2 error (false negative) because the study was adequately powered. Previous reports have suggested that the OR of associated SNP or MBL-producing haplotypes are in the range 2.1–3.1[6,7,9], and our study had 80% power to detect an OR of 2.0 (2.1 for analysis restricted to RA patients) for even the least frequent polymorphism examined at the 5% significance level. The codon 54*G/A SNP did show a trend to association with presence of erosions by 5 years (OR = 1.4, 95% CI: 0.95-2.05, p = 0.09). We calculate, however, that a much larger sample size would be required to confirm an association. Thus, in excess of 1,700 patients, with half having erosive disease by 5 years, would be needed to have 80% power to detect an association at the 5% significance level. All SNP were in Hardy-Weinberg equilibrium with allele frequencies close to those reported previously. Furthermore, our results are in keeping with a previous cross-sectional investigation of 189 Danish patients with longstanding RA, which showed no association of erosion score with *MBL* genotype[11]. Finally, it is interesting to note that in a large study of individuals from Southern China, even allowing for differences in frequencies of *MBL* polymorphisms, serum MBL levels were significantly lower in patients with RA than controls, supporting the hypothesis that polymorphism within *MBL* alone is not accounting for the low MBL levels observed in RA patients[7].

Our results suggest that polymorphism within *MBL* itself does not play a major role in the development of erosions in inflammatory polyarthritis. Hence genotyping patients for these polymorphisms at presentation will not help target more aggressive therapies to patients likely to develop erosions. It does not preclude, however, a role for a gene acting upstream in the MBL pathway resulting in low MBL levels and development of erosions.

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