

Polymorphism in the MHC-encoded LMP7 Gene: Association with JRA without Functional Significance for Immunoproteasome Assembly

SAMPATH PRAHALAD, DANIEL J. KINGSBURY, THOMAS A. GRIFFIN, BOBBI-LYNN COOPER, DAVID N. GLASS, WALTER P. MAKSYMOWYCH, and ROBERT A. COLBERT

ABSTRACT. *Objective.* To determine if a polymorphism in the immunoproteasome subunit LMP7 was associated with juvenile rheumatoid arthritis (JRA) and had functional significance.

Methods. The frequency of LMP7QQ+ vs QQ- (QK and KK genotypes) among 207 patients with JRA and 50 controls was determined. JRA subtypes were pauciarticular (53%), polyarticular (33%), and systemic (14%). Onset was before age 6 (early onset) in 60% of patients. The functional significance of the LMP7 polymorphism was determined by comparing incorporation of LMP7Q vs LMP7K into proteasomes.

Results. There was an increased frequency of LMP7QQ in patients vs controls (73 vs 56%; $p = 0.016$), mainly due to the pauciarticular and systemic JRA subtypes ($p = 0.037$), and more pronounced in early onset disease (77 vs 56%; $p = 0.006$). The association persisted with stratification for HLA-DR5(11) and -DPB1*0201 ($p = 0.002$ and 0.013). We found no difference in the relative incorporation of LMP7Q and LMP7K into proteasomes.

Conclusions. These results support an association between LMP7QQ homozygosity and JRA, particularly early onset disease. The difference persists with stratification, at least for DR5(11) and DPB1*0201, suggesting that this effect is unlikely to be due to linkage disequilibrium with HLA alleles known to be associated with early onset pauciarticular JRA. Importantly, as there does not appear to be functional significance associated with the LMP7 polymorphism, this may be a marker for another as yet unidentified susceptibility locus. (J Rheumatol 2001;28:2320-5)

Key Indexing Terms:

JRA LMP7 PROTEASOME ASSOCIATION LMP2 IMMUNOPROTEASOME

Juvenile rheumatoid arthritis (JRA) encompasses a spectrum of autoimmune arthropathies that vary in presentation, course, extraarticular manifestations, and longterm outcome. Polymorphisms of HLA genes in the major histocompatibility complex (MHC) have been shown to be associated with susceptibility to JRA¹, and more recently linkage between this region and both pauciarticular and polyarticular JRA has been demonstrated^{2,3}. However, JRA is a complex genetic trait and

known polymorphisms do not account for the entire susceptibility, suggesting that other polymorphisms both within and outside the MHC contribute⁴.

The class II region of the MHC includes HLA-DR, -DQ and -DP as well as genes involved in MHC class I antigen processing and presentation, such as those encoding the low molecular weight polypeptides (LMP2 and LMP7) and the transporters associated with antigen processing (TAP1 and TAP2). LMP2 and LMP7 are interferon gamma (IFN- γ)-inducible subunits of a large protease complex known as the proteasome, which is involved in the degradation of intracellular proteins and the generation of antigenic peptides that are presented by MHC class I molecules^{5,6}. Along with a third IFN- γ -inducible subunit MECL-1, LMP2 and LMP7 can substitute for constitutively synthesized homologues (LMP2 for delta, MECL-1 for Z, and LMP7 for X) during proteasome assembly to form immunoproteasomes^{7,8}. This occurs via a cooperative mechanism whereby LMP2 and MECL-1 are preferentially co-incorporated into proteasome assembly intermediates, which subsequently incorporate LMP7 preferentially over X⁹.

LMP7, like all catalytic proteasome subunits, is synthesized with an N-terminal propeptide that is removed autocatalytically upon completion of assembly, exposing the active

From the William S. Rowe Division of Rheumatology, Children's Hospital Medical Center, Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA and the Department of Medicine, University of Alberta, Edmonton, Alberta, Canada.

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S. Prahalad MD, Fellow in Rheumatology; D.J. Kingsbury, MD, Fellow in Rheumatology; T.A. Griffin MD, PhD, Research Instructor in Pediatrics, D.N. Glass, MD, Professor of Pediatrics; R.A. Colbert, MD, PhD, Associate Professor of Pediatrics, William S. Rowe Division of Rheumatology, University of Cincinnati College of Medicine; B. Cooper, Clinical Research Technician; W.P. Maksymowych, MD, Associate Professor of Medicine, Scholar of the Alberta Heritage Foundation for Medical Research, Department of Medicine, University of Alberta.

Address reprint requests to Dr. R.A. Colbert, Division of Rheumatology, Children's Hospital Medical Center, 3333 Burnet Ave., Cincinnati, Ohio, 45229-3039. E-mail: bob.colbert@chmcc.org

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site of the catalytic subunit. This propeptide is also necessary for efficient incorporation of LMP7 into proteasomes, and is largely responsible for the preferential incorporation of this subunit into immunoproteasomes¹⁰. Immunoproteasomes, and LMP7 in particular, may be important in the generation of antigenic peptides. For example MHC class I expression is reduced (about 50%) on lymphoid cells from LMP7-deficient mice¹¹. Whether this effect is due to the absence of LMP7 *per se*, or a result of altered composition of other proteasome subunits as well is not clear.

The LMP7 propeptide is polymorphic, with Lys (K) or Gln (Q) found at position -24. An association between homozygosity for Q at -24 (LMP7QQ) and susceptibility to ankylosing spondylitis (AS) has been reported¹². Given the role of the LMP7 propeptide in immunoproteasome formation and the potential role of LMP7 alleles in susceptibility to arthritis, we sought to determine whether there is an association with susceptibility to JRA, and furthermore whether the propeptide polymorphism affects immunoproteasome assembly.

MATERIALS AND METHODS

Patients and controls. The study population consisted of 207 patients (144 females, 63 males) who met the American College of Rheumatology criteria for JRA and were followed by the Division of Rheumatology at the Children's Hospital Medical Center, Cincinnati. To test for the influence of LMP7 polymorphism on disease phenotype, patients were stratified based on onset type and age of onset. The onset type of JRA was pauciarticular in 110 (53.1%), polyarticular in 68 (32.9%), and systemic in 29 (14%) patients. Onset was before the 6th birthday in 125 patients (60.4%) who were classified as having early onset JRA. Controls were 50 individuals who closely resembled the JRA population with respect to ethnic mix and geographic origin.

LMP7 genotyping. LMP7 genotypes were determined on purified genomic DNA using a PCR-RFLP method. The forward primer was CGGACA-GATCTCTGGGTGCT and the reverse primer was CACTGGCTTCCC-TACTGCC. Cycling was carried out with Taq polymerase at 95°C for 30s, 66°C for 20s, and 72°C for 30s for 30 cycles. Digestion with Bsm I was at 65°C for 3 hours. The resulting DNA fragments were 174 and 25 bp for the Q allele (Gln) and 199 bp for the K allele (Lys). A single nucleotide polymorphism (A for C) in the first position of the codon for amino acid -24 of the propeptide, results in the substitution of Lys (K) for Gln (Q). Thus, the 3 possible allelic combinations are QQ, QK, and KK.

HLA typing. HLA class I and II typing was performed using standard typing sera and microcytotoxicity assays. Because HLA-DR serologic splits were completed in most, but not all patients, the HLA-DR5 and DR11 as well as HLA-DR6 and DR13 were pooled as DR5(11) and DR6(13), respectively. HLA results were available for 203 patients and 49 controls. Data were analyzed using chi-square with one degree of freedom and, where appropriate, Fisher's exact test. SPSS 8.0 for Windows was used for the data analysis.

Cell culture and antibodies. Lymphoblastoid T2 cells (0.174 × CEM^R)¹³ were obtained from P. Cresswell (Yale University, New Haven, CT, USA) and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum as described⁹. MCP21 is a mouse monoclonal antibody obtained from the European Collection of Animal Cell Cultures (Salisbury, Wiltshire, UK) that recognizes a human α -type proteasome subunit (C3) and is able to immunoprecipitate proteasomes and recognize C3 on immunoblots¹⁴. Polyclonal antisera recognizing human LMP2 and LMP7 were obtained from J. Monaco (University of Cincinnati, Cincinnati, OH, USA).

DNA constructs and transfection. Human LMP7 subunits were expressed from cDNA cloned into the episomal vector pCEP4 (hygromycin^r) (Invitrogen, Carlsbad, CA, USA). LMP2 was expressed using pCEP9

(neomycin^r), as described^{9,10}. pCEP9 was constructed in our laboratory and is similar to pCEP4 except that it confers resistance to neomycin rather than hygromycin^{9,10}. LMP7K was made by mutating the codon for amino acid -24 in LMP7Q (CAG) to AAG using the Altered Sites[®] II system (Promega, Madison, WI, USA). Construction of the cDNA encoding LMP7 without its propeptide (Δ PP) and LMP2.His was described previously¹⁰. Δ PP.LMP7 encodes Met (start codon) followed by Thr, the first amino acid residue of the mature LMP7 subunit. LMP2.His encodes the full-length LMP2 subunit with 6 additional C-terminal His residues. All of the cDNA encoding altered subunits were completely sequenced to ensure that no undesired changes were introduced. T2 cells were transfected with cDNA in episomal vectors, and selected with G418 and/or hygromycin, as described⁹.

Immunoprecipitation, nickel precipitation, gel electrophoresis and immunoblotting. Cells were harvested in rapid growth phase and lysed in 20 mM Tris, pH 7.6, 10 mM EDTA, and 100 mM NaCl with 1% NP-40. Immunoprecipitation was performed on post-nuclear supernatants using 1 μ g antibody per 10⁶ cells, followed by protein G-Sepharose (250 μ g/10⁶ cells). Samples were boiled in sodium dodecyl sulfate sample buffer, separated on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted using proteasome subunit-specific antibodies or antisera as described⁹. For visualization, alkaline phosphatase-conjugated goat anti-mouse IgG was used. Nickel precipitation of His-tagged LMP2-containing proteasomes was performed with 100 μ l of Ni-NTA agarose (Qiagen, Hilden, Germany) per 10⁷ cells. Proteasomes were eluted from Ni-NTA agarose with 250 mM imidazole and boiled in 6 × SDS sample buffer prior to SDS-PAGE⁹. Material from 5 × 10⁶ cells was used for LMP7 and C3 immunoblots, while 16 × 10⁶ cells were used for LMP2.

RESULTS

LMP7 QQ genotype associated with clinical subtypes of JRA. In our initial analysis, we compared the frequencies of QQ, QK, and KK. However, because the number of homozygous KK individuals is small, we grouped QK and KK together and report them as QQ-, with homozygosity for Q reported as QQ+. When the entire patient population was compared to controls, we found a statistically significant increase in homozygosity for LMP7Q (73.4 vs 56%; $p = 0.016$) (Table 1). To determine whether this increase was restricted to any particular subgroup of JRA, patients were divided into polyarticular, pauciarticular, and systemic onset, and further subdivided into early and late onset. Although the trend towards

Table 1. Comparisons of the distribution of LMP7QQ among different clinical categories.

Category	QQ+ (%)	QQ-	Total	p Value
All controls	28 (56.0)	22	50	
All patients	152 (73.4)	55	207	0.016
All polyarticular	49 (72.1)	19	68	0.07
Early	19 (79.2)	5	24	0.053
Late	30 (68.2)	14	44	0.23
All pauciarticular	80 (72.7)	30	110	0.037
Early	62 (72.9)	23	85	0.044
Late	18 (72.0)	7	25	0.18
Systemic	23 (79.3)	6	29	0.037
Early	15 (93.8)	1	16	0.006
Late	8 (61.5)	5	13	0.764
All early onset	96 (76.8)	29	125	0.006
All late onset	56 (68.3)	26	82	0.15

increased QQ+ in patients occurred in all subgroups, a statistically significant difference ($p < 0.05$) was seen only in the patients with pauciarticular onset JRA ($p = 0.037$) and systemic onset JRA ($p = 0.037$). Interestingly, when the entire patient group was stratified by age of onset, homozygosity for LMP7Q was most pronounced in the early onset JRA patients ($p = 0.006$ compared to all controls), consistent with an earlier study relating age of onset to HLA polymorphisms¹⁵.

LMP7 QQ genotype frequencies in patients and controls stratified by HLA alleles. To determine whether increased homozygosity for LMP7Q results from linkage disequilibrium with HLA alleles known to be associated with JRA, we stratified patients and controls by HLA type. For the class I allele HLA-A2 that is associated with early onset pauciarticular JRA, there is an excess of LMP7QQ in patients compared with controls (Table 2), although the difference is not statistically significant. For HLA-B27, which is associated with late onset pauciarticular JRA, there were only 3 controls and the difference in the frequency of LMP7QQ was not significantly different. For the class II alleles HLA-DR5(11), -DR8, and -DPB1*0201, which are associated with early onset JRA, again there is an excess of LMP7QQ even with stratification, and for HLA-DR5(11) and -DPB1*0201, the effect is statistically significant ($p = 0.002$ and 0.013 , respectively). Interestingly, when HLA-DR5(11) or -DR8-positive patients are compared with matched controls, the excess of LMP7QQ remains significant suggesting an independent effect of LMP7Q. This is also true even with HLA-A2 included. Twelve of 13 patients who had both HLA-DR5 and -DR8 had LMP7QQ (92.3%). None of the controls had this combination, precluding statistical analysis.

In contrast, stratification for HLA-DR1 and -DR6(13) results in a loss of excess LMP7Q homozygosity, primarily by increasing the QQ frequency in controls (to 69.2–75%). For HLA-DR4, excess LMP7Q homozygosity remains, although the difference is not significant. It appears that, at least for the HLA alleles strongly associated with pauciarticular JRA such as -A2, -DR5(11), -DR8, and -DPB1*0201, there may be an independent effect of LMP7QQ that is not due to linkage disequilibrium.

Lack of functional significance of the LMP7Q/K polymorphism. Using a cell line that lacks endogenous LMP2 and LMP7, we studied the co-dependence of these subunits for incorporation into immunoproteasomes⁹, and defined the role of the LMP7 propeptide in this process¹⁰. When LMP7 is expressed it is efficiently incorporated into proteasomes in place of its constitutive homologue X, whether or not LMP2 is present. In contrast, LMP2 is incorporated efficiently and replaces its constitutive homologue delta only in the presence of LMP7. Although LMP7 does not require LMP2 for incorporation, greater incorporation is seen when these subunits are co-expressed owing to the formation of 2 proteasome populations, one containing delta/LMP7 and the other containing

LMP2/LMP7¹⁰. These observations are shown in Figure 1A, and have been reported previously^{9,10}. Importantly, the LMP7 propeptide plays a critical role in facilitating both LMP7 and LMP2 incorporation. As shown in Figure 1A, when the LMP7 propeptide is truncated (Δ PP lane), very little LMP7 or LMP2 is incorporated into proteasomes¹⁰.

We next compared the incorporation of LMP7Q and LMP7K, which differ only at position -24 of the propeptide (Figure 1B). We found no difference between the amount of mature LMP7 incorporated into proteasomes in cells expressing LMP7Q vs LMP7K (Figure 1B). This experiment was repeated several times with multiple independent transfections, and no consistent differences were observed. We also compared the relative amount of LMP7 incorporated into proteasomes when LMP2 is co-expressed. Again, there is no apparent difference in LMP7 incorporation when LMP7Q or LMP7K is expressed (Figure 1C), nor is there any difference in the amount of LMP2. Since LMP7 is incorporated into 2 populations of proteasomes in the presence of LMP2 (i.e., “immunoproteasomes” with LMP2, and those with delta), we looked for differences in the distribution of LMP7. To do this we engineered LMP2 with a polyhistidine tag (LMP2.His), which enables isolation of LMP2.His-containing proteasomes using nickel-agarose¹⁰, with subsequent immunoprecipitation of remaining proteasomes containing delta. We have found that 2 rounds of nickel precipitation removes > 95% of the histagged proteasomes¹⁰ (data not shown). Using this methodology we found that immunoproteasomes containing LMP2.His isolated from T2 cells expressing LMP7Q or LMP7K have the same amount of LMP7 (Figure 1C, Ni lanes, Q vs K). Furthermore, the remaining delta-containing proteasomes immunoprecipitated with MCP21 contain the same amount of LMP7 when cells express LMP7Q or LMP7K (Figure 1C, IP lanes, Q vs K). Immunoblots for LMP2 confirmed its presence in the nickel precipitates and absence from the MCP21 immunoprecipitates (data not shown). Therefore, at least from the standpoint of proteasome assembly, there does not seem to be any functional significance of the Q/K polymorphism at position -24 of the propeptide.

DISCUSSION

We investigated whether the polymorphism in exon 2 of the LMP7 gene, which alters the sequence of the LMP7 propeptide, is associated with JRA. Our results demonstrate that LMP7QQ is significantly associated with early onset JRA. This effect does not appear to be due to linkage disequilibrium with HLA class II alleles known to be associated with JRA, as evidenced by the persistence of the effect when compared against HLA matched controls with HLA-DR5, -DPB1*0201, or either -DR5 or -DR8. The presence of a recombination hotspot in the MHC class II region between LMP7 and HLA-DR further reduces the likelihood of linkage disequilibrium between these genes^{16,17}. However, it should be noted that the HLA typing methods employed for our study

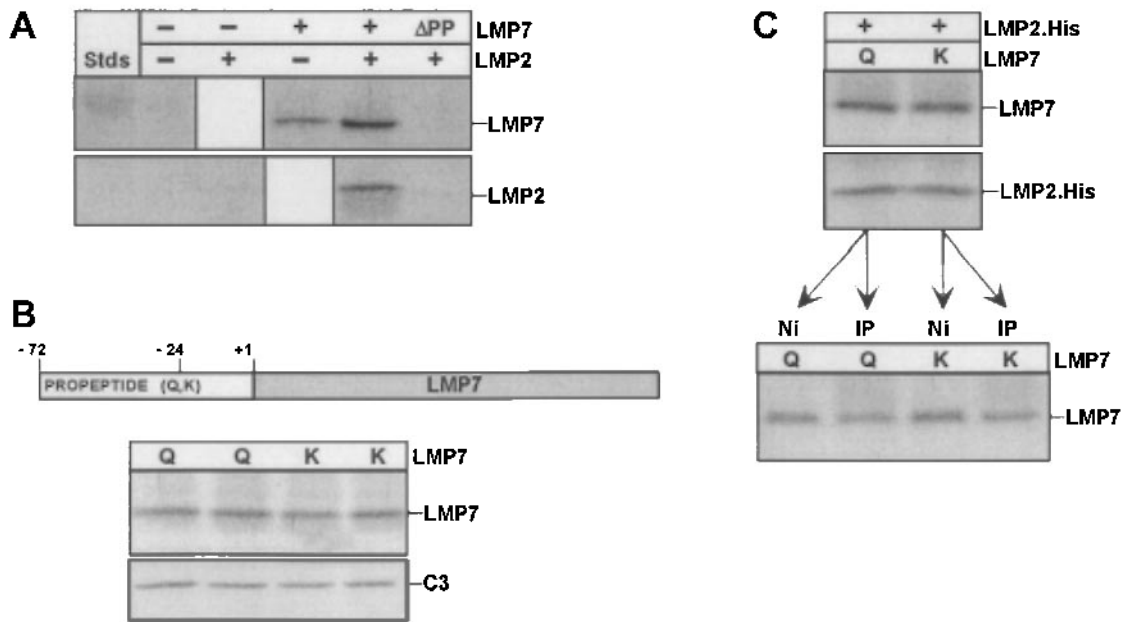


Figure 1. The effect of LMP7Q/K polymorphism on LMP7 and LMP2 incorporation into proteasomes. (A) Proteasomes were immunoprecipitated from untransfected T2 cells, and T2 expressing LMP2 or LMP7 alone, or together. Proteasome subunits were separated by SDS-PAGE, and LMP7 and LMP2 visualized by immunoblotting with respective antisera. Pre-stained molecular weight standards (Stds) were run in the first lane, and a band at 28.5 kDa is visible on the LMP7 blot. (+) or (-) indicates whether the cells are expressing the subunit shown at the right. LMP7 expressed without its propeptide is designated DPP. (B) Top: Schematic of the full-length LMP7 subunit with the Q/K polymorphism at position -24 of the propeptide is shown. The mature subunit is 204 amino acids in length. Bottom: Proteasomes were immunoprecipitated from T2 cells expressing either LMP7Q (Q) or LMP7K (K), and LMP7 visualized as above. Two independent cell lines expressing LMP7Q and 2 with LMP7K are shown. The C3 immunoblot demonstrates that approximately the same amount of proteasomes were recovered and loaded for each sample. (C) Top: Proteasomes were immunoprecipitated from T2 cells expressing LMP2.His and either LMP7Q (Q) or LMP7K (K), and subunits visualized as described above. Bottom: In a parallel experiment, LMP2.His-containing immunoproteasomes were first separated by 2 successive rounds of nickel precipitation, followed by immunoprecipitation of the remaining proteasomes with MCP21. Material from the first round of nickel precipitation (Ni) and the MCP21 immunoprecipitation (IP) is shown.

are low resolution, and consequently cases and controls may not be matched completely despite stratification. Thus we cannot completely eliminate the possibility that linkage disequilibrium contributes to the LMP7 association.

A similar association between LMP7 polymorphism and AS has been demonstrated by Fraile, *et al*, who investigated 57 patients with AS and 102 random controls from Spain and found that LMP7QQ was present significantly more frequently in patients compared with controls (100 vs 89%; $p = 0.011$)¹². This excess persisted even when patients and controls were stratified for HLA-B27 positivity. However an association has not been found between this LMP7 polymorphism and RA^{18,19}. Similarly, an association between this polymorphism and sarcoidosis or Behçet's disease was not found in Japanese patients²⁰.

It should be noted that our controls have a lower frequency of LMP7QQ compared with other published studies (56 vs 89% of controls reported by Fraile, *et al*¹² and 72% reported by Maksymowych, *et al*¹⁸). Interestingly, this is also true in our patient population (about 73 vs 100% of AS patients of Fraile, *et al*¹² and 82% of RA patients reported by Maksymowych, *et al*¹⁸, although this difference is not as great). Since our entire population is derived from the Midwestern USA it is likely to differ in ethnicity from popu-

lations studied previously in Spain¹² and Western Canada¹⁸. The controls chosen for our study were randomly selected from a pool of unrelated healthy individuals ethnicity-matched to our study population. To ensure that the randomly selected controls were representative of the larger group, the frequencies of several other HLA class I and class II alleles, including HLA-A2, -B27, -DR1, -DR4, -DR5, -DR6 and -DR8 were compared. We found no significant difference between the controls used in this study and 254 controls reported by Murray, *et al*¹⁵ (data not shown). Thus, it seems unlikely that our controls differ immunogenetically from the larger pool, and it is probable that the frequency of LMP7QQ varies in different racial and ethnic groups. This is not surprising as it is encoded in the MHC, which displays considerable polymorphism with various allele frequencies differing around the world.

Another polymorphism occurs on intron 6 of the LMP7 gene²¹. Deng, *et al* reported that this intronic polymorphism was strongly associated with insulin-dependant diabetes mellitus, independent of linkage disequilibrium to HLA or the closely linked TAP2 genes²¹. A third polymorphism has been observed at position -65 (Arg for Gly) of the LMP7 propeptide, but it is rare, with an allele frequency of < 1% in healthy Caucasians (Maksymowych, *et al*: unpublished observations).

Table 2. Comparisons of LMP7QQ frequencies stratified by HLA alleles.

Category	Patients			Controls			p Value
	QQ+	%	Total	QQ+	%	Total	
HLA A2+	97	71.6	135	15	57.7	26	0.15
HLA B27+	12	75.0	16	3	100	3	0.33
HLA DR1+	30	68.2	44	3	75.0	4	0.78
HLA DR4+	27	65.9	41	6	42.9	14	0.13
HLA DR5(11)+	49	85.4	59	5	41.7	12	0.002
HLA DR6(13)+	37	64.9	57	9	69.2	13	0.77
HLA DR8+	35	82.6	41	3	60.0	5	0.16
HLA DPB1*0201	45	76.3	59	4	36.4	11	0.013
HLA DR5(11)/8+	71	82.6	86	8	47.1	17	0.002
HLA A2+ DR5(11)/8	55	85.9	64	5	50.0	10	0.007

In studies similar to those reported here, this polymorphism does not appear to affect proteasome assembly (unpublished observations).

Associations have been demonstrated between a polymorphism in the LMP2 gene and AS, as well as JRA. In one study, HLA-B27 positive AS patients with acute anterior uveitis and/or peripheral arthritis revealed significant differences in genotypic distribution of LMP2 compared with HLA-B27 positive patients with only spinal arthritis, or with HLA-B27 positive controls. This suggests that homozygosity for the LMP2 B allele (BB) is associated with the presence of acute anterior uveitis and peripheral arthritis in HLA-B27 positive patients with AS²². However, Burney, *et al* did not find differences in LMP2 allele or genotype frequencies between patients with AS and either random or HLA-B27 matched controls²³. In a cohort of patients with JRA, LMP2BB was associated not only with increased susceptibility in certain subgroups of JRA, but was also felt to influence the phenotype of the disease, predisposing to a more progressive and severe articular disease in HLA-B27 positive patients²⁴. However LMP2 polymorphisms were found to have no association with susceptibility or outcome in patients with adult RA^{18,19}.

Despite the various associations described between LMP2 polymorphisms and disease, functional studies that implicate a mechanism for how these gene products affect disease have been lacking. The proteasome has an important role in the generation of antigenic peptides that are displayed by MHC class I molecules. The LMP2 polymorphism occurs in the mature protein and could conceivably affect cleavage rate or specificity, and thus the repertoire of antigenic peptides. The LMP7 propeptide plays a vital role in the formation of immunoproteasomes as well as the delta/LMP7 proteasome subset, and thus could also affect antigen processing. However, from the standpoint of proteasome assembly, there does not appear to be an effect of the Q/K polymorphism in the propeptide. We have not examined whether this polymorphism affects the incorporation of MECL-1 into immunopro-

teasomes. However, this seems unlikely given the observed co-dependence of LMP2 and MECL-1²⁵.

We cannot rule out the possibility, although remote, that some other as yet undiscovered function of the propeptide exists. In addition, since peptides derived from the degradation of the full-length propeptide are potentially presented by MHC class I molecules, this sequence difference could affect processing and presentation of such peptides. Given the lack of evidence to support functional significance of this polymorphism, the association of LMP7QQ with both AS and JRA supports the idea that this may be a marker for another non-HLA susceptibility allele in the MHC that has yet to be identified as associated with inflammatory arthritis. This would not be surprising, as the MHC is one of the most densely packed regions of the human genome with about 40% of the expressed genes estimated to have immune system functions²⁶. Results from the genome-wide scan being undertaken in JRA will improve our ability to further define genes involved in susceptibility to inflammatory arthropathies.

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