PSMB8 (LMP7) but not PSMB9 (LMP2) gene polymorphisms are associated to pigeon breeder’s hypersensitivity pneumonitis

Ángel Camarena a, Arnoldo Aquino-Galvez a, Ramcés Falfán-Valencia a, Gloria Sánchez a, Martha Montaño a, Carlos Ramos a, Armida Juárez a, Carolina García-de-Alba a, Julio Granados b, Moisés Selman a,*

a Instituto Nacional de Enfermedades Respiratorias “Ismael Cosio Villegas”, Tlalpan 4502, Sección XVI, CP 14080, México City, DF, Mexico

b Instituto Nacional de Ciencias Médicas y Nutrición “Salvador Zubirán”, Vasco de Quiroga 15, Sección XVI, 14080 México City, Mexico

Received 20 November 2009; accepted 20 January 2010
Available online 11 February 2010

KEYWORDS
Hypersensitivity pneumonitis; PSMB9; LMP2; PSMB8; LMP7

Summary
Hypersensitivity Pneumonitis (HP) is a lung inflammatory disorder caused by inhalation of organic particles by a susceptible host. However, only a small proportion of individuals exposed to HP-associated antigens develop the disease, suggesting that additional host/environmental factors may play a role. We have previously found that genetic susceptibility associated to the major histocompatibility complex (MHC) plays an important role in this disease. The low molecular weight proteosome (LMP, currently named PSMB) genes code for subunits of the proteosome, a multimeric enzymatic complex that degrades proteins into peptides in order to be presented in the MHC class I pathway. We hypothesized that polymorphisms in PSMB8 or PSMB9 genes could be involved in the susceptibility to HP. Thus, in this study we analyzed the polymorphic site at amino acid position 60 (Arg/His) of the fourth exon in the PSMB9 gene and the amino acid position 49 (Gln/Lys) in the second exon of PSMB8 gene in 50 Mexican patients with HP and 50 healthy ethnically matched controls. PSMB typing was performed using polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP). Our results demonstrated that HP patients had a significant increase of the PSMB8 KQ genotype frequency (OR = 7.25, CI = 2.61–21.3; p = 0.000034). No differences were found in the distribution of PSMB9 alleles/genotypes.

* Corresponding author. Tel.: +52 55 5487 1771; fax: +52 55 5665 4623.
E-mail address: mselman@yahoo.com.mx (M. Selman).
Introduction

Hypersensitivity pneumonitis (HP) is an immunologically mediated lung disease caused by repeated inhalation of a wide variety of antigens in susceptible individuals. Pigeon breeder’s disease, one of the most common forms of HP, is provoked by exposure to various avian-derived antigens. The clinical presentation is heterogeneous and the individuals exposed to low levels of avian antigens, can develop sub-acute or chronic forms of the disease and some of them may evolve to pulmonary fibrosis. Importantly, only a small proportion of exposed individuals develop the disease indicating that susceptibility involves genetic or other environmental factors. We have previously identified some haplotypes of the major histocompatibility complex (MHC) class II and polymorphisms in the transporter associated with antigen processing (TAP) genes associated with increased risk to develop HP.

The antigen processing machinery is the combination of the cellular processes responsible for the presentation of endogenous peptides by HLA class I molecules. These peptides are generated by specialized immunoproteasomes composed of LMP2, LMP7 and LMP10 subunits. The LMP2 and LMP7 genes are located within the class II region, and encode two subunits of the proteasome complex involved in the degradation of cytosolic proteins and the generation of antigenic peptides. Recently, the Human Genome Organization (HUGO) Gene nomenclature committee and the HLA informatics group established the actual name for LMP2 (Proteasome [prosome, macropain] subunit, beta type, 9 [large multifunctional peptidase 2]) and for LMP7 as PSMB8 (Proteasome [prosome, macropain] subunit, beta type, 8 [large multifunctional peptidase 7]). The analysis of these gene polymorphisms have been typically performed by using codon positions (LMP2 Arg60His and LMP7 Gln49Lys); however, this nomenclature makes difficult the comparison of alleles and genotypes frequencies in international databases. We use "rs number" for evaluation and comparison of our results (Table 1 summarizes the previous and current nomenclature).

The aim of this study was to investigate whether polymorphisms in PSMB8 and PSMB9 genes are involved in the susceptibility to hypersensitivity pneumonitis.

Study population

Fifty unrelated patients with diagnosis of HP provoked by avian antigens (pigeon breeder’s disease, 10 males and 40 females) were included in this study. Diagnosis of HP was established as described elsewhere. Briefly, all patients had home exposure to birds and positive serum antibodies against avian antigens determined by ELISA. Chest high-resolution computed tomography scanning showed diffuse centrilobular poorly defined micronodules, ground glass attenuation, focal air trapping and mild/moderate fibrotic changes, and the bronchoalveolar lavage displayed >40% lymphocytes. Forty-five percent of the patients were biopsied and histopathological abnormalities confirmed the diagnosis of HP. The control group (34 males, 16 females) was composed by fifty ethnically matched unrelated healthy Mexican bird-exposed but asymptomatic subjects (EAS), without history of collagen-vascular disorders, diabetes or other autoimmune or chronic-degenerative disease. Patients and controls had at least two generations that were born in Mexico, and thus considered to be Mexican Mestizo. The protocol was approved by the Scientific and Bioethic Committee and patients and controls signed an informed consent letter.

Genomic DNA extraction

Genomic DNA was extracted from peripheral blood using a BDtract genomic DNA isolation kit (Maxim Biotech, San Francisco CA).

Amplification of genomic DNA

To obtain the sequences that include the polymorphic sites, we designed two pairs of primers: (5’-GGC AGC AAC GGA CAG CCC AAC ATG-3’; 5’-GTA CGA GGC GAA AGC C-3’) for PSMB9 and (5’-CGG ACA GTC TCT TGG GTG CT-3’; 5’-CTC CCG GGA CTG AAG GCT A-3’) for PSMB8. All PCR amplifications were performed on 40 ng of genomic DNA in a 25 μL reaction volume containing 50 mM KCl, 10 mM TrisCl, pH 8.3, 1.5 mM MgCl2; 200 μM of each dNTP’s and 20 pmol of each primer. Samples were subjected to 1 hold 96 °C 5 min

Table 1: Actual and previous nomenclature of PSMB9 and PSMB8 polymorphisms.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Polymorphisms</th>
<th>Exon</th>
<th>Codon</th>
<th>Residue Change</th>
<th>Position</th>
<th>SNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSMB9</td>
<td>LMP2</td>
<td>4</td>
<td>CGC → CAC</td>
<td>R (Arg) → H (His)</td>
<td>60</td>
<td>rs17587</td>
</tr>
<tr>
<td>PSMB8</td>
<td>LMP7</td>
<td>2</td>
<td>CAG → AAG</td>
<td>Q (Gln) → K (Lys)</td>
<td>49</td>
<td>rs2071543</td>
</tr>
</tbody>
</table>

HGNC: HUGO Gene Nomenclature Committee (11).
and then linked to 94 °C 60 s, 64 °C 60 s and 72 °C 30 s, 30 cycles for PSMB8, and 94 °C 60 s, 60 °C 60 s and 72 °C 60 s, 25 cycles for PSMB9; this cycling was followed by a final extension at 72 °C for 7 min, using an automated thermal cycler (GeneAmp PCR system 9700, PE Applied Biosystems, Foster City CA). Amplifications were performed using Platinum Taq DNA polymerase (Invitrogen, Brazil). Amplification PCR products were electrophoresed in submarine 2% agarose gels containing 0.2 μg/mL ethidium bromide for 40 min (30 V/cm) and the amplified bands were visualized in a dual intensity UV light trans-illuminator (UVP Inc., Upland, CA), and finally analyzed and stored in EDAS290 photodocumentation system (Kodak, Rochester NY).

Restriction of amplified products

The polymorphic site at amino acid position 60 (Arg/His) of the fourth exon in the PSMB9 gene and the amino acid position 49 (Gln/Lys) in the second exon of PSMB8 gene, were analyzed by Restriction Fragment Length Polymorphism (RFLP) method. The PCR products 252 for PSMB9 and 304 for PSMB8 were digested with 7 U Cfo1 and 4 U Bsm1 (New England Biolabs, Beverly, MA) respectively, at 37 °C for 4 h. Since Cfo1 recognizes the polymorphic site for PSMB9, the H (His) allele was demonstrated by the presence of two fragments (210 and 42 bp) whereas the R (Arg) allele was revealed by the presence of a single 252 bp band. Alleles of PSMB8 were defined by Bsm1, where Q (Gln) had two bands of 174 and 130 bp, and K (Lys) a single 304 bp band. The obtained fragments were electrophoresed in a 3% agarose 1000 gel and visualized by ethidium bromide staining.

Statistical analysis

Allele and genotype frequencies of PSMB9 and PSMB8 were determined by direct counting in both study groups. Association between specific PSMB9 and PSMB8 was assessed by Fisher’s Exact Tests at conventional significance levels (<0.05) and odds ratios (OR) and their corresponding 95% confidence intervals (CI), using Epi Info 6 v 6.04d statistical program (Stone Mountain, GA). Association analysis was done at allele, genotype and haplotype level.

Results

Allele and genotype frequencies

We analyzed PSMB9 and PSMB8 polymorphisms in 50 HP patients and 50 bird-exposed but asymptomatic individuals by using RFLP. This strategy was preferred instead of the hybridization with Sequence Specific Oligonucleotides (SSO) and Sequence Specific Primers (SSP) because it is a potent tool to detect polymorphisms and make false positive results very unlikely. Also, false negative data are avoided and the homozygosity of the sample tested is unequivocally established.

The allele frequencies (af) of the PSMB9 and PSMB8 genes in the amino acid position 60 and 49, respectively are shown

<table>
<thead>
<tr>
<th>Allele</th>
<th>HP patients</th>
<th>EAS group</th>
<th>p Value</th>
<th>OR (CI 95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 50</td>
<td>n = 50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSMB9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>68</td>
<td>65</td>
<td>0.764</td>
<td>1.14 (0.61–2.15)</td>
</tr>
<tr>
<td>H</td>
<td>32</td>
<td>35</td>
<td>0.764</td>
<td>0.87 (0.46–1.64)</td>
</tr>
<tr>
<td>PSMB8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q</td>
<td>53</td>
<td>80</td>
<td>0.000098</td>
<td>0.28 (0.14–0.55)</td>
</tr>
<tr>
<td>K</td>
<td>47</td>
<td>20</td>
<td>0.000098</td>
<td>3.55 (1.82–7.03)</td>
</tr>
</tbody>
</table>

HP: hypersensitivity pneumonitis; EAS: exposed asymptomatic subjects; OR: Odds ratio, CI: confidence interval, af: allele frequency.

Restriction of amplified products

The polymorphic site at amino acid position 60 (Arg/His) of the fourth exon in the PSMB9 gene and the amino acid position 49 (Gln/Lys) in the second exon of PSMB8 gene, were analyzed by Restriction Fragment Length Polymorphism (RFLP) method. The PCR products 252 for PSMB9 and 304 for PSMB8 were digested with 7 U Cfo1 and 4 U Bsm1 (New England Biolabs, Beverly, MA) respectively, at 37 °C for 4 h. Since Cfo1 recognizes the polymorphic site for PSMB9, the H (His) allele was demonstrated by the presence of two fragments (210 and 42 bp) whereas the R (Arg) allele was revealed by the presence of a single 252 bp band. Alleles of PSMB8 were defined by Bsm1, where Q (Gln) had two bands of 174 and 130 bp, and K (Lys) a single 304 bp band. The obtained fragments were electrophoresed in a 3% agarose 1000 gel and visualized by ethidium bromide staining.

Statistical analysis

Allele and genotype frequencies of PSMB9 and PSMB8 were determined by direct counting in both study groups. Association between specific PSMB9 and PSMB8 was assessed by Fisher’s Exact Tests at conventional significance levels (<0.05) and odds ratios (OR) and their corresponding 95% confidence intervals (CI), using Epi Info 6 v 6.04d statistical program (Stone Mountain, GA). Association analysis was done at allele, genotype and haplotype level.

Results

Allele and genotype frequencies

We analyzed PSMB9 and PSMB8 polymorphisms in 50 HP patients and 50 bird-exposed but asymptomatic individuals by using RFLP. This strategy was preferred instead of the hybridization with Sequence Specific Oligonucleotides (SSO) and Sequence Specific Primers (SSP) because it is a potent tool to detect polymorphisms and make false positive results very unlikely. Also, false negative data are avoided and the homozygosity of the sample tested is unequivocally established.

The allele frequencies (af) of the PSMB9 and PSMB8 genes in the amino acid position 60 and 49, respectively are shown

<table>
<thead>
<tr>
<th>Allele</th>
<th>HP patients</th>
<th>EAS group</th>
<th>p Value</th>
<th>OR (CI 95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 50</td>
<td>n = 50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSMB9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RH</td>
<td>24</td>
<td>19</td>
<td>0.419</td>
<td>1.51 (0.63–3.61)</td>
</tr>
<tr>
<td>RR</td>
<td>22</td>
<td>23</td>
<td>1.0</td>
<td>0.92 (0.39–2.18)</td>
</tr>
<tr>
<td>HH</td>
<td>4</td>
<td>8</td>
<td>0.355</td>
<td>0.46 (0.09–1.87)</td>
</tr>
<tr>
<td>PSMB8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KQ</td>
<td>29</td>
<td>8</td>
<td>0.000034</td>
<td>7.25 (2.61–21.3)</td>
</tr>
<tr>
<td>QQ</td>
<td>12</td>
<td>36</td>
<td>0.000004</td>
<td>0.12 (0.05–0.33)</td>
</tr>
<tr>
<td>KK</td>
<td>9</td>
<td>6</td>
<td>0.575</td>
<td>1.61 (0.46–5.98)</td>
</tr>
</tbody>
</table>

HP: hypersensitivity pneumonitis; EAS: exposed asymptomatic subjects; OR: Odds ratio, CI: confidence interval, gf: genotype frequency.
in Table 2. Regarding PSMB8, the K (Lys) allele was significantly increased in the HP group with an allele frequency of 0.47 versus 0.20 in the EAS group (OR = 3.55; 95% CI = 1.82–7.03; p < 0.0001). Accordingly, the frequency of the Q (Gln) allele was decreased in the HP patients. In contrast, allele analysis of PSMB9 did not reveal significant differences among patients and control subjects.

The evaluation of the genotypes showed a significant increase of the heterozygous KQ genotype in HP in comparison with EAS controls (OR = 7.25 95% CI = 0.2.61–21.3; p < 0.000034) (Table 3). Likewise, the frequency of the genotype QQ was considerably lower in HP group (OR = 0.12 95% CI = 0.05–0.33). Regarding PSMB9, RH heterozygous was the most common genotype in HP patients (gf = 0.48), while RR homozygous prevailed in EAS group (gf = 0.46). However, no associations were found between patients and exposed healthy subjects. All genotypes were under Hardy–Weinberg Equilibrium (HWE).

**Haplotype analysis**

Haplotype frequencies are presented in Table 4. A total of seven haplotypes PSMB9-PSMB8 were detected in both groups. The HH-KQ combination was present only in the EAS group while HH-KQ was found only in the HP patients group. In the EAS, two haplotypes gather 64% of combinations (RH-KQ 62% and RR-QQ 26%). The distribution in HP patients was heterogeneous but two haplotypes reach 42% of combinations (RH-KQ = 22% and RR-KQ = 20%). The haplotype RH-KQ was significantly increased in the HP group when compared to EAS controls (OR = 6.77, 95% CI = 1.34–65.31; p < 0.02). Likewise, HP patients displayed a decreased frequency of haplotype RH-QQ (p < 0.001, OR = 0.14 95% CI = 0.03–0.49).

**Discussion**

We have previously found that genetic susceptibility associated to MHC class II as well as to variants of the TAP genes are implicated in the risk to develop the disease. In the present study we found that polymorphisms of the PSMB8 gene appear also to be involved. Proteasomes are distributed throughout eukaryotic cells at a high concentration and cleave foreign peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway. The PSMB9 and PSMB8 coding genes are located within the class II region, and participate in the antigen processing.

In this context, putative associations between PSMB genes and a large variety of autoimmune, infectious and inflammatory diseases have been reported. In this study, we explored the distribution of polymorphisms in the PSMB9 and PSMB8 genes corresponding to amino acid positions 60 and 49 respectively, in patients with hypersensitivity pneumonitis and exposed but asymptomatic Mexican Mestizo subjects. Our results revealed a significant increase of K allele and KQ genotype of PSMB8 gene, as well as the haplotype RH-KQ in the HP patients. Although the functional relevance of these SNPs in HP is yet unclear, the strong associations point to either direct gene or near loci to influence HP. HLA-independent effects of PSMB gene polymorphisms have been associated to other diseases like diabetes mellitus.

There are no studies determining whether the products of different PSMB alleles differ with respect to the peptides produced. However, these subunits may directly affect peptide cleavage specificity which may be important to determine susceptibility in immunopathological disorders such as hypersensitivity pneumonitis. Some evidence emerges from mice models, where preliminary findings suggest that structural polymorphism in LMP2 (Arg60His) may have functional implications. Interestingly, a single amino acid change at position 272 from glycine to arginine significantly affects its migration on SDS-PAGE gels. Moreover, in mutant mice a target deletion of the LMP gene resulted in the decreased expression of MHC class I molecules on cell surface, which leads to inefficient endogenous antigen presentation.

It has been demonstrated that proteasomes containing LMP7 (PSMB8) play an indispensable role in the survival of mice infected with *Toxoplasma gondii*, presumably due to the efficient generation of cytotoxic T lymphocytes epitopes required for the functional development of CD8(+) T-cells. Activation of cytotoxic T-cells play a relevant role in the pathogenesis of HP and requires presentation of endogenous antigenic peptides by MHC class I molecules. In this context, it is currently accepted that an exaggerated cell-mediated immune response contributes to the development of HP.

Interestingly, it was recently reported that a selective inhibitor of PSMB8 (PR-957) blocks LMP7-specific
presentation in MHC class I restricts antigens in vitro and in vivo. As a consequence, this inhibition prevents the production of interleukin-23 (IL-23) by activated monocytes and interferon-γ and IL-2 by T lymphocytes. In mice rheumatoid arthritis models, PR-957 treatment reversed signs of disease and resulted in reductions of cellular infiltration, cytokine production and autoantibody levels. These studies reveal a unique role for LMP7 in controlling pathogenic immune responses and provide a therapeutic rationale for targeting LMP7 in autoimmune disorders.

Finally, we have reviewed the alleles frequencies of these SNPs in different populations (Table 5): African, Amerindians of Brazil and Mexico, Asian, Caucasians, Hispanic (Spain) Mexican ancestry from Los Angeles people, Mexican Mestizo populations and the data obtained in our study, and we found that PSMB8 alleles and genotype frequencies are similar among populations with the exception of native Amerindian groups where low frequency of PSMB8 K alleles are observed, while the Kaingang population (Brazil) showed a high frequency of this allele. This result suggest that this allele was absent in humans from northern Asia who settled in the Artic and populated the Americas more than 25,000 years before and that it has been acquired more recently by genetic admixture. Regarding to PSMB9 gene we observed differences between the analyzed groups, displaying heterogeneity in their distribution particularly when comparing with the Amerindian population. Some genetic populations studies support the notion that distribution of PSMB and TAP alleles in people living in developed countries may differ from that seen in people living in developing ones due to pathogen driving selection pressures. These findings suggest that the differences in frequencies of PSMB9 genotype could be helpful in distinguishing each of these populations.

In summary, the results of this study suggest that there is an association between some genotype of the PSMB genes and hypersensitivity pneumonitis in Mexican population. Particularly PSMB8 KQ genotype may play a role in the development of HP while PSMB QQ might play a protective role. Additional studies in a large cohort of patients and functional analyses of individual SNPs could help to establish the real significance and deleterious effects of these variations located in the coding region of LMP gene in the susceptibility to HP.

### Conflict of interest statement

The authors have no conflicts of interest to declare.

### References


