Restriction Fragment Length Polymorphism Analysis in the HLA Class III Genes of Patients with Diffuse Panbronchiolitis

Yasuyuki Tomita, Shu Hashimoto, Takako Shimizu, Kazue Son, Arata Azuma*, Shoji Kudoh* and Takashi Horie

Although diffuse panbronchiolitis (DPB) is known to be positively associated with certain major histocompatibility complex (MHC) class I antigens, e.g., HLA-B54 in Japanese patients, it is not clear whether the MHC genes predispose to the disease or are markers for other disease susceptibility gene(s). Because the HLA class III genes such as tumor necrosis factor (TNF) or the fourth component of complement (C4) are localized in the proximity of the HLA-B locus, one or more of these genes might be responsible for susceptibility to DPB. To analyze the role of HLA class III genes in DPB patients, we first evaluated the HLA-B54 association in 32 patients with DPB, and subsequently, studied the restriction fragment length polymorphism (RFLP) of the TNF-α and -β (TNF-α/β) genes as well as the C4A and B (C4A/B) genes in DPB patients and normal individuals. The HLA-B54 antigen was significantly more frequent in DPB patients than in normal individuals (40.3% vs 13.0%, p<0.001), however, we did not detect a significant association between DPB and gene polymorphisms of either TNF-α/β or C4A/B. Furthermore, there was no evidence of C4A gene deletion in patients with DPB. These results suggest that the HLA-B54 antigen itself might be directly involved in the pathogenesis of DPB.

Key words: HLA-B54, tumor necrosis factor (TNF) genes, fourth component of complement (C4) genes

Introduction

Diffuse panbronchiolitis (DPB) is an obstructive lung disease seen almost exclusively in Japan (1). This disorder is characterized by chronic recurrent sinopulmonary infection, respiratory bronchiolitis, and peribronchiolitis, which are disseminated diffusely throughout the bilateral lungs, particularly in the lower lobes. Although the precise etiology of DPB remains unknown, familial occurrence has been recognized, suggesting that this disease may have a genetic background (1, 2).

Furthermore, a population study has shown that DPB is positively associated with certain HLA class I antigens, such as B54 in Japanese patients (3). Despite this association, the role of this molecule in DPB is not clear. It is even questionable whether the HLA genes predispose toward the disease or are markers for other closely linked genes.

Recent cytogenic studies have demonstrated that the genes for human tumor necrosis factor -α and -β (TNF-α/β) (4) as well as the fourth component of complement A and B (C4A/B) (5) are located within the HLA class III region, close to the HLA-B locus (Fig. 1). Therefore, these findings led us to the hypothesis that not only the HLA-B54 gene but also HLA class III genes, such as TNF-α/β or C4A/B, might be responsible for susceptibility to DPB.

To test this hypothesis, we examined restriction fragment length polymorphism (RFLP) analysis to the TNF-α/β and C4A/B genes using restriction endonucleases in peripheral blood mononuclear cells (PBMC) from patients with DPB.

Materials and Methods

Patients

Thirty-two patients who satisfied the diagnostic criteria of DPB according the Japanese Ministry of Health and Welfare (6) were subjected to HLA class I typing. Twenty-three of 32
patients underwent RFLP analysis of TNF-α/β genes, and 9 of 32 underwent RFLP analysis of C4A/B genes. All patients in this study also had the following clinical features: 1) typical radiologic features on chest film; 2) chronic paranasal sinusitis; and 3) chronic coughing and sputum discharge.

**HLA typing**

HLA typing for antigens A, B, and C was performed using the standard lymphocyte microcytotoxicity test (7).

**Cell preparation and DNA extraction**

PBMC from DPB patients and normal individuals were separated by gradient centrifugation of heparinized venous blood on Ficoll-Hypaque (Litton Bionetics, Kensington, MD) at 1,500 rpm for 30 minutes. The cells were washed three times in PBS and then genomic DNA was extracted by previously described methods (8).

**Preparation of DNA probes**

The 0.3 kb BstE III/Ava I fragment of human TNF-α cDNA and the 0.8 kb BamH I/EcoR I fragment of human TNF-β cDNA were kindly provided by the Suntory Institute of Biology (Osaka, Japan) (9). The 0.5 kb BamH I/Kpn I fragment specific for the 5' ends of both C4A and B genes was derived from the full length C4 cDNA clone pAT-A (American Type Culture Collection; Rockville, MD) (10).

One hundred nanograms of purified cDNA was radiolabeled by random primer extension in the presence of 32P-dCTP. The resultant specific activity was approximately $7 \times 10^6$ cpm/mg which was used at $2 \times 10^7$ cpm/blot.

**Southern hybridization**

Fifteen microgram samples of DNA were digested with 50 units each of the restriction endonucleases, BamH I, Hind III, Nco I, and Taq I (New England Biolabs, Beverly, MA) under conditions recommended by the supplier. The samples were subjected to overnight electrophoresis at 20 V on 0.7% agarose gels in TBE (Tris/boric acid/EDTA) buffer. DNA was then transferred to nylon membranes (Hybond N+, Amersham, UK) by Southern transfer. The blots were pre-hybridized for 15 minutes at 65°C and then hybridized for 3 hours at 65°C by Rapid Hybridization Buffer (Amersham) with a 32P-labeled cDNA probe. After hybridization, the blots were washed with 0.1 x SSC/0.5% SDS for 20 minutes at 65°C and exposed to XAR-5 X-ray film (Eastman Kodak Co., Rochester, NY) with intensifying screens for 16 hours at -70°C.

**Statistical analysis**

p-values were calculated by Fisher's exact test.

**Results**

**HLA-B54 association with DPB**

The haplotypes of HLA-A, -B, and -C antigens were determined in 32 patients with DPB and in 100 normal individuals by standard microcytotoxicity assays. As shown in Fig. 2, the HLA-B54 antigen was significantly more frequent in patients with DPB than in normal individuals (40.3% vs 13.0%, p<0.001), consistent with a previous report by Sugiyama et al (3). No significant differences were found in the frequencies of HLA-A and -C antigens between DPB patients and normal individuals (data not shown).

**RFLP analysis of TNF-α/β genes**

Webb and Chaplin (11) described that digestion of genomic DNA with the restriction endonuclease Nco I and Southern blotting with TNF-α cDNA probe shows restriction fragments of 5.5 kb and 10.5 kb, and that the 5.5 kb fragment is associated with the presence of a single Nco I restriction site within the 3'
untranslated region of the TNF-α gene and the first intron of the
TNF-β gene (Fig. 3A). Therefore, we analyzed RFLP of the
TNF-α/β genes in 23 patients with DPB and in 23 normal
individuals, using Nco I and Southern blotting. Although Nco
I-digested DNA revealed a polymorphic pattern, consisting of
restriction fragments of 5.5 kb and 10.5 kb (Fig. 3B), and these
fragments were identical when Southern hybridization was
carried out with either the TNF-α or -β cDNA probe (data not
shown), the allelic frequencies of the 5.5 and 10.5 kb alleles
were not significantly different between DPB patients and
normal individuals (Table 1). Furthermore, no significant
difference was found in the frequencies of the 5.5 and 10.5 kb
alleles between B54-positive and -negative patients with DPB
(data not shown).

RFLP analysis of C4A/B genes
RFLP of C4 A/B genes was analyzed in 9 DPB patients and
8 normal individuals by Southern hybridization using the 5' C4
pAT-A probe (0.5 kb BamH I/Kpn I fragment) and three
restriction endonucleases, Hind III, BamH I, and Taq I. As
described in other reports, the C4B gene can exist in either a
“short” or “long” form depending on the absence or presence,
respectively, of a 6.5 kb intron located approximately 3 kb
downstream from the 5' end of C4B. The patterns of Taq I-, Hind
III-, and BamH I-restriction fragments were described previously
by Olsen et al (12).

Table 1. Gene Frequencies in Nco I-TNF RFLP
Analysis from Patients with DPB and Normal Individuals

<table>
<thead>
<tr>
<th>TNF allele (% frequency)</th>
<th>Total</th>
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<tr>
<td>5.5 kb</td>
<td></td>
</tr>
<tr>
<td>10.5 kb</td>
<td></td>
</tr>
<tr>
<td>Normal (n=23)</td>
<td></td>
</tr>
<tr>
<td>10 (22)</td>
<td>36 (78)</td>
</tr>
<tr>
<td>DPB (n=23)</td>
<td></td>
</tr>
<tr>
<td>11 (24)</td>
<td>35 (76)</td>
</tr>
</tbody>
</table>

Figure 3. A) Molecular map of TNF-α/β region illustrating
the Nco I restriction fragment pattern. The positions of the Nco I
sites in the TNF region are shown relative to the gene sequences.
Also shown are the locations of the 5.5 kb and 10.5 kb TNF-Nco I
restriction fragments as previously described by Webb and Chaplin
(11). B) Southern blot analysis of NcoI-digested DNA hybridized
with a TNF-α cDNA probe in patients with DPB. Genomic DNA in
PBMC from DBP patients were digested with Nco I and then
hybridized with a TNF-α cDNA probe by Southern blotting.
 Autoradiograph showed two distinct fragments, consisting of 5.5
kb and 10.5 kb (also see Fig. 3A).

Figure 4. Molecular map of the C4 region illustrating the
Taq I-, Hind III-, and BamH I-restriction fragment patterns in the
normal and C4A deletion states. The C4B gene can exist either in
a “short” or a “long” form depending on the absence or presence,
respectively, of a 6.5 kb intron located approximately 3 kb down-
stream from the 5' end of C4B. The patterns of Taq I-, Hind
III-, and BamH I-restriction fragments were described previously
by Olsen et al (12).
Virtually unknown to physicians in Western countries (14). In Korea (13) and Taiwan, a few cases have been reported outside Asia— notably in Asian emigrants— but the disease is virtually unknown to physicians in Western countries. DPB was first described in Japan in 1969 and subsequently in Japanese patients with DPB and normal individuals. Furthermore, Sugiyama et al (3) reported that the frequency of the HLA-B54 antigen is strongly increased in 38 DPB patients (phenotype frequency: 63.2% in DPB vs 11.4% in control) and that B54 exists only in Japanese, Chinese, and Korean populations. These findings suggest that the disease may have a genetic background and racial susceptibility. We performed HLA analysis in 32 patients with DPB and found that the B54 antigen was significantly more frequent in DPB patients than in normal individuals (40.3% vs 13.0%, p<0.001). This is consistent with the above-mentioned report by Sugiyama et al (3).

Two hypotheses can be offered to explain the association between DPB and this particular HLA haplotype. The first hypothesis suggests that the HLA antigen itself is directly involved in the disease process. Because the association between DPB and the HLA-B54 haplotype is relatively weak, this hypothesis remains controversial. The second hypothesis suggests that an additional DPB susceptibility gene(s) is located in the proximity of the HLA-B locus.

In view of the physical distance between HLA class III loci such as TNF or C4, and the HLA-B locus (4, 5), abnormal TNF and/or C4 genes could be a candidate for governing susceptibility to DPB. Therefore, RFLP analysis was carried out to study the possible role of TNF or C4 gene polymorphism in DPB. However, in our sample of normal individuals, we did not find any difference in the allelic frequencies of the 5.5/10.5 kb alleles in NcoI-TNF Southern blot hybridization between DPB patients and normal individuals.

With regard to TNF gene polymorphism, NcoI-TNF RFLP analysis has been reported in several autoimmune diseases. Bettinotti et al (15) recently revealed in a large scale examination that the 5.5 kb allele is significantly increased in Caucasian patients with systemic lupus erythematosus (SLE), as has been also described by us in Japanese patients with SLE (16). Similar results have been found in a study of patients with type I diabetes mellitus (17).

Furthermore, Webb and Chaplin (11) reported that the 5.5 kb fragment is associated with the presence of a single Nco I restriction site within the 3' untranslated region of the TNF-α gene and the first intron of the TNF-β gene, and that substitution of base A to G at nucleotide position 1,069 within the first intron of the TNF-β gene results in the absence of the Nco I site. Messer et al (18) revealed that the amino acid sequence for 5.5 kb allele homozygotes differs from that for 10.5 kb allele homozygotes at amino acid position 26 in the TNF-β gene. They also demonstrated that the 5.5 kb allele is strongly associated with increased TNF-β production by PBMC stimulated with PHA. Although the reason for the failure to detect a positive association between the 5.5 kb allele in Nco I-TNF RFLP and DPB is not clear, TNF gene polymorphism may not be responsible for susceptibility to DPB. Alternatively, the increased frequency of the 5.5 kb allele seen in SLE patients may be secondary to a positive association with a particular HLA haplotype that is in positive linkage disequilibrium with the 5.5 kb allele, since it has been established that SLE is associated with HLA-B8/DR3 in Caucasian patients and with HP. 5.5 kb allele homozygotes differs from that for 10.5 kb allele homozygotes at amino acid position 26 in the TNF-β gene. They also demonstrated that the 5.5 kb allele is strongly associated with increased TNF-β production by PBMC stimulated with PHA. Although the reason for the failure to detect a positive association between the 5.5 kb allele in Nco I-TNF RFLP and DPB is not clear, TNF gene polymorphism may not be responsible for susceptibility to DPB. Alternatively, the increased frequency of the 5.5 kb allele seen in SLE patients may be secondary to a positive association with a particular HLA haplotype that is in positive linkage disequilibrium with the 5.5 kb allele, since it has been established that SLE is associated with HLA-B8/DR3 in Caucasian patients and with HLA-B54.

Table 2. Restriction Fragment Length Patterns in the C4A and C4B Genes of Patients with DPB and Normal Individuals.

<table>
<thead>
<tr>
<th>DPB</th>
<th>C4A</th>
<th>C4B long</th>
<th>C4B short</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>BamHI 4.8 kb</td>
<td>TaqI 7.0 kb</td>
<td>HindIII 26 kb</td>
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<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>8</td>
<td>+</td>
<td>+</td>
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</table>

Restriction fragment length patterns were defined by Southern blot analysis using 0.5 kb BamH I/Kpn I fragment of human C4 cDNA and restriction endonucleases, BamH I, Taq I, and Hind III.

downstream from the 5' end of C4B (12). Hind III-digested DNA revealed 2 RFLP bands, consisting of restriction fragments of 26 kb for the C4B “long”, and 10 kb for the C4B “short”. BamH I-digested DNA had a 4.8 kb fragment for C4B “long” and a 3.3 kb fragment for C4B “short” (Fig. 4). As summarized in Table 2, all DPB patients and normal individuals had the C4B “long” gene, detected by Hind III-26 kb and BamH I-4.8 kb fragments, in addition to the normal C4A gene, detected by Taq I-7.0 kb and BamH I-4.8 kb fragments. Furthermore, 5 of 9 DPB patients and 5 of 8 normal individuals had the C4B “short” gene, detected by BamH I-3.3 kb fragments. Therefore, there were no differences between DPB patients and normal individuals in the RFLP pattern of C4A/B genes. Furthermore, neither DPB patients nor normal individuals had a large C4A, 21-OHA deletion (12), which is detected by loss of the normal C4A 7.0 kb fragment and the presence of a new 6.4 kb fragment in Taq I-digested DNA (Fig. 4 and Table 2).

Discussion

DPB was first described in Japan in 1969 and subsequently in Korea (13) and Taiwan; a few cases have been reported outside Asia— notably in Asian emigrants— but the disease is virtually unknown to physicians in Western countries (14). In
HLA-DR2 in Japanese patients (19).

The HLA class III region contains the genes for three complement proteins (C2, C4 and factor B), which occur in the sequence of C2, factor B, C4A, and C4B. The steroid 21-hydroxylase gene (21-OH) is mapped at <2kb from the 3’ ends of both the C4A and C4B genes and these genes are tandemly arranged in an apparent gene duplication event (5). The C4 genes are thought to be highly polymorphic, based on the following evidence: The C4B gene can exist either in a “short” or a “long” form depending on the absence or presence, respectively, of a 6.5 kb intron located approximately 3 kb downstream from the 5’ end of C4B. Furthermore, Taq I- and Hind III-RFLP analyses reveal a large C4A, 21-OHA deletion (20). Therefore, we performed C4A/B gene-RFLP analysis to investigate the possible role of these alleles in disease susceptibility to DPB. However, we did not find any specific band in our DPB patients when the 5’ C4 pAT-A probe and 3 different restriction endonucleases, Hind III, BamHI, and Taq I, were used. Furthermore, the deletion of the C4A gene, which is judged by the loss of the normal C4A 7.0 kb fragment and by the presence of a new 6.4 kb fragment in Taq I-digested DNA, was not found in our DPB patients.

Deletion of the C4A gene has been linked to susceptibility to a number of diseases, such as SLE (21), autoimmune hepatitis (AH) (22), and idiopathic membranous nephropathy (IMN) (23) in Caucasians or black Americans. Goldstein et al (21) found an increased frequency of C4A gene deletion in Caucasian SLE patients compared with normal individuals. In a study of black Americans, Olsen et al (12) found that 19 of 79 (24%) SLE patients compared with 5 of 68 (7.4%) controls had a C4A gene deletion. Moreover, it has been reported in a study for Caucasians that both AH and IMN patients have more frequent C4A gene deletion than controls (54% vs 13%, 63% vs 13%, respectively). However, many studies have shown strong positive linkage disequilibrium between C4A gene deletion and the extended A1, B8, DR3 haplotype in Caucasians, which is quite rare in Japanese. Therefore, C4A gene deletion seen in these diseases may be secondary to a positive association with a particular HLA haplotype. In fact, the C4A gene deletion was not found in 59 patients with SLE (24) and was found in only one of 31 patients with IMN (23) in studies of Japanese.

In conclusion, we showed a positive association between DPB and the HLA-B54 antigen. However, we did not find a significant association between DPB and TNF gene polymorphism, as detected by Nco I-RFLP analysis, or C4 gene polymorphism, as revealed by Hind III-, BamHI I- and Tag I-RFLP analyses. Furthermore, there was no evidence of C4A gene deletion in patients with DPB, or in normal individuals. These findings suggest that the HLA antigen itself might be directly involved in the pathogenesis of DPB and/or in promoting the progression of this disease.

References


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