Analysis of Human Leukocyte Antigen HLA-DR,β Amino Acid Sequence in Vogt-Koyanagi-Harada Syndrome

Jiro Numaga,* Kazumasa Matsuki,† Katsushi Tokunaga,† Takeo Juji,† and Manabu Mochizuki‡

Vogt-Koyanagi-Harada syndrome (VKH) is a systemic disorder involving many organ systems, including the eye, the ear, the skin, and the meninges. One of the important clinical features of the disease is its strong association with histocompatibility antigens HLA-DR4 and HLA-Dw15. To investigate this association at the DNA level, the polymerase chain reaction technique was used. The DRβ 1 DNA sequence was analyzed to determine which part played the most significant role in the disease process. Although the number of patients studied was small, these data showed that these probes were useful in investigating the HLA-DR subtypes related to VKH. All but one patient (19 of 20) had a specific sequence encoding amino acids 70 and 71 of the HLA-DRβ 1 molecule. The data suggest that the sequence that encodes for amino acids 70 and 71 and adjacent ones of the HLA-DRβ 1 molecule could contribute to disease susceptibility. Invest Ophthalmol Vis Sci 32:1958–1961, 1991

Vogt-Koyanagi-Harada syndrome (VKH) is a bilateral uveitis affecting the choroid, iris, and ciliary body.1–4 In addition to the eye, this disorder affects many systemic organs containing melanocytes, such as the ear, the meninges, the hair, and the skin. The clinical features of the disorder are characterized by acute inflammation of those tissues in its acute stage and depigmentation of the tissues in its late chronic stage. The disorder has a predilection for people of pigmented races and is rare in whites.5,6 Although the cause of the disorder is still unknown, accumulating data suggest that an autoimmune mechanism against melanocytes is involved in the pathophysiology of the disease.7,8

One of the interesting features of VKH is its strong association with the human leukocyte antigen (HLA) system.9–11 Previous serologic and cytologic studies show its strong association with HLA-DR4 and HLA-Dw15.11–12 However, no investigation at the DNA level has been reported thus far to our knowledge.

In this study, we used the newly developed technique of polymerase chain reaction (PCR) and investigated which part of the DR4/Dw15 DRβ 1 DNA sequence plays a significant role in this disorder.

Materials and Methods

Subjects

The patients were 20 unrelated Japanese patients with VKH. They were selected randomly from the outpatients at the Departments of Ophthalmology, Tokyo University Hospital and Tokyo University Branch Hospital. The controls were 47 randomly selected, healthy, unrelated Japanese patients. All studies were approved by the Ethics Committee of Tokyo University, Tokyo, Japan, and written consent was obtained from all subjects before participation.

Methods

Conventional serologic HLA typing was done for 10 of 20 patients with VKH to reconfirm the association with HLA-DR4. For HLA typing, a modified two-stage complement-dependent microcytotoxicity method was used.

Genomic DNA was extracted from the blood as described previously.13 Oligonucleotides were synthesized by the methoxy phosphoramidite method synthesizer. The primers used were GLPDRβ 1 (5’-TTCTTCTCAATGGGACGGAG-3’) and GAMPDRβ 1 (5’-GCGGCTGCAGTGAGCTCT-3’).14 The GLPDRβ 1 anneals to the sequence encoding amino acids 17–23 of the DRβ molecules; GAMPDRβ 1 is complementary to the sequence for amino acids 87–94. Three probes were used in this study. They all anneal to the exon 2 of the HLA-DR4/Dw15 DRβ 1 genes.14–17 Probe 1 (5’-TACTTCAATCACCAAGAGGA-3’) anneals to the DR4 DRβ 1 that encodes amino acids 30–36. This sequence is found in all the known DR4 DRβ 1 sequences (Fig. 1). Probe 2 (5’-

From the *Department of Ophthalmology, Tokyo University School of Medicine, †Blood Transfusion Service, Tokyo University Hospital, and the ‡Department of Ophthalmology, Tokyo University Branch Hospital, Tokyo, Japan.

Submitted for publication: April 20, 1990; accepted February 4, 1991.

Reprint requests: Jiro Numaga, MD, 3-2-1-214, Nishigahara, Kita-ku, Tokyo 114, Japan.
CGGCCTAGCGCCAGTTAC-3’) anneals to the sequence that encodes amino acids 55-60. This sequence is found only in DR4/Dw15 DRβ1 and DRw8 DRβ1 sequences. Probe 3 (5'-GCAGAGCGGGCCGCGGT-3’) anneals to the sequence that encodes amino acids 70-75. This sequence is found in DR4/Dw14 DRβ1, DR4/Dw15 DRβ1, and DR1 DRβ1.

The PCR was done as described by Saiki and co-workers \(^\text{18}\) with modifications. We used 100 µl of the reaction mixture containing 1 µl of genomic DNA, 50 mM Tris HCl (pH 8.8), 10 mM MgCl₂, 10 mM ammonium sulfate, 1.5 mM deoxyadenosine triphosphate, 1.5 mM deoxycytidine triphosphate, 1.5 mM deoxyguanosine triphosphate, and 1.5 mM deoxythymidine triphosphate, and 0.5 µl of each primer. The samples were heated at 95°C over 5 min and left at room temperature for 0.5 min. After the addition of 2 units of Taq DNA polymerase and 40 µl of mineral oil, the sample tubes were incubated at 55°C for 1.5 min, at 72°C for 1 min, and at 95°C for 1 min. The cycling was repeated 25 times. After the last cycle, all samples were incubated for an additional 5 min at 72°C to complete the extension.

After PCR amplification, 30-µl samples were removed and underwent electrophoresis to confirm the amplification and evaluate the amount of amplified DNA. The remaining samples (70 µl) were mixed with 1 ml of 0.5M NaOH and 25 mM ethylenediaminetetraacetic acid (EDTA) and incubated for 5 min at room temperature. Aliquots (200 µl) of the mixture were slot blotted to a nylon filter. The filters were baked at 80°C for 2 hr and stored at room temperature until use.

The hybridization and prehybridization were done at 46°C, 50°C, and 55°C for probes 1, 2, and 3, respectively. Each probe hybridized specifically to the complementary sequence at that temperature. The filters were prehybridized in fivefold concentrated sodium/sodium phosphate/EDTA (5 X SSPE, ie, 0.9 M NaCl, 0.05 M sodium biphosphate, 5 mM EDTA, pH 7.4), fivefold concentrated Denhardt’s solution, and 0.5% sodium dodecyl sulfate (SDS) for 1 hr at the hybridization temperature. The oligonucleotide probe (10 pmol) was 3'-end labeled by [α-32P] Dideoxyadenosine triphosphate (ddATP) (3000 cpm) and purified over a Sephadex-G25 column (Pharmacia, Sweden). After hybridization, the filters were washed once in twofold concentrated SSPE for 5 min at room temperature, then twice in twofold concentrated SSPE with 0.5% SDS for 10 min at the hybridization temperature. The filters were exposed for periods ranging from 1 hr to overnight at -70°C with two intensifying screens (Dupont, USA).

Results

The results of serologic HLA typing were as follows. All ten patients were DR4 positive. Twelve subjects among the 47 controls (26%) were DR4 positive. Figure 2 shows the agarose gel of representative am-
Fig. 2. Agarose gel electrophoresis showing representative amplified products. Lane 1 shows size marker (DNA/Hae III). Lanes A–G represent the PCR products from seven patients. Arrow shows amplified products (231 bp).

amplified products from seven patients with VKH disease. All samples had strongly amplified products with a molecular size of approximately 231 base pairs.

Figure 3 shows the representative blot samples from patients with VKH. Thus, among 20 patients, 15 were positive for probe 1, 11 for probe 2, and 19 for probe 3 (Table 1). The rate of positive reactions to each of the three probes in VKH patients was significantly higher than that in the controls. Among them, probe 3 showed the highest relative risk; the subjects with the sequence that hybridized with probe 3 had a 55.4 times greater risk than those without it. Lower risk was observed for probe 2.

**Table 1. Reaction of the probes to the patients with VKH**

<table>
<thead>
<tr>
<th>Probes</th>
<th>Patients (n = 20)</th>
<th>Controls (n = 47)</th>
<th>Relative risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe 1 (DR4)</td>
<td>15 (75%)</td>
<td>12 (26%)</td>
<td>8.8†</td>
</tr>
<tr>
<td>Probe 2 (Dw15, DRw8)</td>
<td>11 (55%)</td>
<td>12 (26%)</td>
<td>3.6*</td>
</tr>
<tr>
<td>Probe 3 (Dw14, Dw15, DR1)</td>
<td>19 (95%)</td>
<td>12 (26%)</td>
<td>55.4‡</td>
</tr>
</tbody>
</table>

* P < 0.05, †P < 0.001, ‡P < 0.000001.

Discussion

To our knowledge, this is the first study to investigate the association between the HLA system and VKH at the DNA level. Previous studies on this disease have been limited to conventional serologic or cytotactic techniques. They documented strong associations of this disease with HLA-DR4 and HLA-Dw15. In our study, the strong association with HLA-DR4 was also shown in the ten patients tested by serologic HLA typing, although we were unable to type in other ten patients. No previous studies have used the techniques of molecular biology. This is partly because of the extreme similarity of the HLA Class II DNA sequences among the DR4 subtypes. No difference in the DRα sequence has been reported thus far. The DRβ4 sequence (which encodes the DRw53 specificity) is also identical among the DR4 subtypes. Even the DRβ1 sequences have only minor differences. Such minor deviation cannot be detected by conventional methods such as analysis of restriction fragment length polymorphism. However, the newly developed technique of PCR enabled us to dissect minor nucleotide differences precisely.

From the viewpoint of the relative risk, the most important sequence in developing this disease is that detected by probe 3 (Table 1), which codes amino acids 70–75 of the DR4DRβ1 chain. According to a recent model for the three-dimensional structure of HLA Class II antigen, the antigen-binding portion of HLA protein is composed of two α helices and one β sheet. The antigenic peptide is most likely caught in a cleft between the α helices, and T-cells recognize the antigen together with the HLA molecule. In this model, amino acids 70 and 71, located at the inner top portion of the α helix, are postulated to bind the antigen or T-cell receptor molecule directly. Our hypothesis is that this portion of the HLA molecule affects its binding to various antigen molecules or T-cell receptors influencing the immune response to foreign and autologous peptides, and consequently determines the susceptibility of the individual to this disorder. The second important sequence was detected by probe 1. This sequence encodes amino acids 30–36 of the DR4DRβ1 chain. Amino acid 30 is located on the floor of the cleft. This site is also postulated to play a role in antigen binding and thus could assist in the disease process.
Because the number of the patients tested was small, our results should be considered preliminary. However, this study demonstrates that PCR can be used to define the HLA subtypes related to VKH. Furthermore, it raises the possibility that an epitope composed of amino acids 70 and 71 on HLA-DRβ1 molecules could play an important role in susceptibility to VKH among the Japanese population.

Key words: HLA, HLA-DRβ chain, amino acid sequence, Vogt-Koyanagi-Harada syndrome, polymerase chain reaction (PCR)

Acknowledgments
The authors thank Mr. Masami Itoh for his excellent technical assistance.

References