structure of this 12.5-kDa protein reported here has yet to be clarified.

In our study, elevated amounts of a 12.5-kDa protein were observed in 56% of PSX eyes. It is unclear why this elevation was not observed in 44% of the PSX eyes but was found in 1 of 59 eyes without clinical signs of PSX. Possible explanations include that PSX might be a heterogeneous disease process with expression of characteristic signs in only a disease subgroup, or that the accumulation of the 12.5-kDa protein is not directly related to the PSX disease process. Interestingly, neither the accumulation of the 12.5-kDa protein nor the increase of total aqueous protein appeared to be correlated with presence or absence of secondary glaucoma capsular. Therefore, neither the impairment of the blood–aqueous barrier nor the presence of the 12.5-kDa band appears to be directly related to the development of secondary OAG in PSX.

Recently, typical PSX material have been identified at several extraocular and systemic locations. Therefore, further elucidation of protein alterations related to the development of secondary OAG in PSX.

Methods. HLA-DR4 gene variations were investigated in 46 Japanese patients, 28 with the prolonged type and 18 with the nonprolonged type of VKH. HLA-DR4 genes were amplified with polymerase chain reaction (PCR) and then analyzed for its variation with single-strand conformation polymorphism (SSCP) and restriction fragment length polymorphism (RFLP) methods.

Results. Significant differences were found in the DR4 gene variation in the two clinical subtypes. All the patients with the prolonged type had either the DRB1*0405 or DRB1*0410 variant, whereas 39% of the patients with the nonprolonged type had neither of them. This difference in frequency was statistically highly significant ($P = 0.00059, PC = 0.0041$). DRB1*0405 was also more frequent in the prolonged type (93%) than in the nonprolonged type (56%) ($P = 0.0044, PC = 0.030$). In the prolonged type, relative risk was highest for DRB1*0405/0410 (128), whereas in the nonprolonged type it was highest for DR4 (8.6).

Conclusion. This preliminary study showed that DR4 gene variants differed significantly between the two clinical subtypes of Vogt-Koyanagi-Harada disease (VKH).
from the generalized disruption of normal retinal architecture (with peripheral retinal rosettes) that was seen in the photosensitized eyes. It is likely that the lack of RPE in association with the ectopic retinal tissue at the optic nerve head was responsible for the rosette formation in the controls.

There is considerable variability in the pathology produced by photosensitization because no single eye manifested the entire spectrum of changes. There are several possible reasons for this variability. First, the beagle is born with a relatively opaque lens that significantly attenuates the transmission of short wavelengths (blue) of visible light while permitting relatively greater transmission of longer wavelengths. This opacity gradually clears over the first 2 weeks after birth, but the rate of clearing varies by a few days from pup to pup. Therefore, depending upon the lens clarity at the time of treatment, the amount of light reaching the retina will vary among the pups. Moreover, the lens opacity precluded examination of the fundus by indirect ophthalmoscopy. Thus, the early events of the disease process could not be observed. Finally, there may be inherent variability in the disease itself. In human ROP, the majority of stage 1 and stage 2 lesions regress without any residual evidence of disease. Regression may account for the failure to observe microscopically the ridge and shunt vessels noted 1 to 2 weeks before enucleation in a few of the treated eyes. This variability could also account for the lack of a clear correlation between duration of exposure and severity of retinal damage. The most severe retinal vascular damage was seen in eye IV-G treated for 45 minutes, but the eyes treated for 25 or 35 minutes showed less severe changes than eye IV-B treated for 15 minutes, which showed total retinal detachment.

Oxygen1 and light2 have been studied as potential etiologic factors in the development of ROP. Photosensitization provides the mechanism by which these two factors may interact in the presence of a photosensitizer to generate reactive oxygen species. Rose bengal photoreactions have been shown to generate singlet oxygen,17-19 and PP IX photooxidation has been shown to yield both singlet oxygen and superoxide.20 Photostimulization has been established as a potential mechanism of retinal injury. Gottsch et al have proposed hematogenous photosensitization by PP IX to be perhaps an important process in the development of age-related macular degeneration and have demonstrated deposits in Bruch’s membrane secondary to PP IX photosensitization in mice.21 Wilson et al have shown that RB-mediated photosensitization in the retina of adult animals (rats, cats, rabbits) can damage endothelial cells, leading to thrombus formation and vascular occlusion.22 The developing retinal vasculature of a preterm infant, which is relatively deficient in protective antioxidants,6 may be even more sensitive to such damage. Injury to endothelial cells in the immature retina may range from subtle damage to obliteration and closure of nascent retinal vessels (these changes have already been described as features of the kitten23 and rat24 models of ROP). This vascular occlusion with associated retinal ischemia may be the precipitating event in these retinopathies.

We propose that increasing any of the three components of the photosensitization mechanism (oxygen, light, photosensitizer) could potentially increase the production of reactive oxygen and the degree of subsequent retinal injury. The level of the hematogenous photosensitizer, PP IX, has been found to be elevated in preterm infants10 as well as in neonatal beagle puppies. There is also considerable evidence that preterm infants may receive a higher retinal light dose than their older counterparts. The high dependency areas of neonatal units (where the most premature infants are kept) have been found to be significantly brighter than the low dependency areas.32 Moreover, Robinson and Fielder have reported that the eyelid closure reflex to light does not develop until 28 to 31 weeks gestation,34 and the pupillary response to light does not appear until 30 weeks.35,36 Hence, preterm neonates younger than this age will have larger pupillary diameters and presumably greater retinal irradiance (as well as higher photosensitizer levels) than older neonates; thus, they will be at risk for increased free radical production and retinal injury. Our model predicts that reducing the level of one factor, such as oxygen tension, may not necessarily be enough to prevent retinal injury; the photosensitizer levels and the amount of light exposure may be adequate to compensate for the reduced oxygen tension and still generate sufficient free radicals to produce retinal damage. This would explain the continued incidence of ROP among very low birthweight preterm infants (who would presumably have higher photosensitizer levels and larger retinal light dose) despite more restricted use of oxygen.37 It is noteworthy that in our beagle model, we were able to generate retinal injury in a normoxic environment. A similar explanation may also account for the failure to observe a reduction in the incidence of ROP with reduced light exposure in the study by Ackerman et al38 (the lower level of light may still have been sufficient for photosensitization injury to occur). Glass et al, however, did find a reduction in the incidence of ROP in infants protected from light.39

There is mounting evidence that ROP is a multifactorial disease, and numerous risk factors in addition to light and oxygen—such as repeated blood transfusions, xanthine administration, vitamin E deficiency, gestational age–birth weight, and patent ductus arteriosus—have been reported.39-41 A number of these
subtypes of VKH, suggesting that the clinical course of VKH is determined partly by the patient’s HLA-DR gene variation. Invest Ophthalmol Vis Sci. 1994;35:752–756.

Vogt-Koyanagi-Harada Disease (VKH) is a bilateral, diffuse, granulomatous uveitis often associated with alopecia, vitiligo, poliosis, tinnitus, dysacusis, and meningeal symptoms.\(^\text{1,2}\) The disease is characterized by an acute inflammation of the melanocyte-containing tissues with eventual depigmentation and is especially prevalent among pigmented races. The disease is classified into two clinical subtypes, nonprolonged and prolonged, depending on the course of inflammation.\(^\text{1,2}\) Though the exact cause of the disease is unknown, autoimmune mechanisms have been suggested in the pathogenesis of the disease.\(^\text{1–3}\)

One of the important features of this disease is its strong association with HLA-DR4.\(^\text{3}\) The HLA-DR4 specificity, originally defined by serology, is now considered to include at least 12 gene variants, DRB1*0401 through DRB1*0412.\(^\text{4}\) Although our previous study\(^\text{3}\) identified a possible DRB1 sequence motif associated with VKH, there have been no reports concerning which variant of HLA-DR4 is related to VKH, nor, more important, concerning whether these variants have any relationship to clinical features. In this study, we investigated the HLA-DR4 gene variants in two clinical subtypes of VKH using recently developed techniques of molecular biology, and we found that DR4 gene variant frequencies differed significantly between these groups.

**MATERIALS AND METHODS.** Patients. The patients (21 men and 25 women) entered into this study were outpatients at the Tokyo University Hospital, Tokyo University Branch Hospital, Omiya Red-Cross Hospital, and Asahi Central Hospital. Twenty of them were included in the previous study.\(^\text{3}\)

All patients underwent complete ophthalmologic and related examinations. All of them had received systemic steroids according to doses recommended by Masuda et al.\(^\text{5}\) Clinical subgrouping was made according to Mimura and colleagues,\(^\text{1}\) namely, patients with prolonged VKH were defined as those in whom inflammation persisted for more than 6 months or in whom it relapsed after 6 months in spite of proper steroid therapy. Patients with nonprolonged VKH were defined as those in whom inflammation subsided within 6 months and did not recur. According to these criteria, 28 patients (16 men and 12 women) had the prolonged type, and 18 (5 men and 13 women) had the nonprolonged type.

Controls were 218 randomly selected, apparently healthy Japanese blood donors at the Saitama Medical Center. All studies were approved by the Ethics Committee of the University of Tokyo, and informed consent was obtained from each patient before participation.

**HLA Analysis.** HLA-DR4 gene typing was carried out by polymerase chain reaction (PCR) amplification,\(^\text{6}\) followed by single-strand conformation polymorphism (SSCP)\(^\text{7}\) and restriction fragment length polymorphism (RFLP) analyses.\(^\text{8}\) Genomic DNA was extracted from the peripheral white blood cells with phenol–chloroform extraction.\(^\text{6}\) Then DNA was amplified by PCR using DR4-specific primers, DRBAMP-4 (5'-GTTTTCTTGAGCAAGGTAAAC) and DRBAMP-B (5’-CCGCTGCACCTGAAAGCTCT), at 94°C 30 seconds, 61°C 30 seconds, and 74.0°C 45 seconds for 30 cycles with DNA Thermal Cycler (Perkin Elmer, Norwalk, CT).\(^\text{6}\)

The SSCP analysis was performed according to Orita and colleagues,\(^\text{2,7}\) with modifications. Briefly, 3 μl of PCR amplified product was mixed with 20 μl loading buffer (95% formamide, 20 mM EDTA/pH 8.0, 0.05% bromphenol blue, 0.05% xylene cyanol) and heated-denatured at 95°C for 5 minutes. Then 3 μl of this mixture was applied on a 8% polyacrylamide gel (acylamide: bis = 49: 1 with 5% glycerol). We included reference DNAs from known DR4 cell lines in every gel. Electrophoresis was performed at 25°C to 30°C for 3.5 to 4 hours at 30 mA in 45 mM Tris/pH 7.6, 45 mM boric acid, 1 mM EDTA (0.5x TBE). The gel was stained with the silver staining kit (Bio-Rad, Richmond, CA) after electrophoresis according to manufacturer’s recommendation.

PCR-RFLP was performed according to Ota and colleagues.\(^\text{8}\) Briefly, 8 μl of PCR-amplified sample was incubated with 5 μl of restriction endonuclease (Sac II, Hinf I, Ava II, Hae II, Mnl I, or Hph I) in 20 μl of appropriate buffer at 37°C overnight. After digestion, 10 μl of the mixture was run on a 8% polyacrylamide gel. Hae III digested pX174 was used as a molecular weight marker.

**Statistical Analysis.** Fisher’s exact test was used to obtain P values for comparison between the two clinical subgroups.\(^\text{9}\) Corrected P values (Pc) was calculated as

\[
Pc = 1 - (1 - P)^n
\]

where n stands for the number of DR4 gene variants observed in the compared groups.\(^\text{9}\) Relative risk was calculated as \((a \times d)/(b \times c)\), where \(a, b, c,\) and \(d\) stand for the numbers of marker+ patients, marker—patients, marker+ controls, and marker— controls, respectively.\(^\text{8}\) When any cell contained 0, we applied Haldane’s modification, in which .5 is added to every number.\(^\text{9}\)

**RESULTS.** Figure 1 shows a representative gel of the SSCP analysis. Most alleles were easily distinguished by a single SSCP run, although DRB1*0404

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and DRB1*0406 were difficult to distinguish by a single run. However, even in such cases, the RFLP analysis (Fig. 2) and multiple SSCP runs with different running temperatures could unambiguously assign the DR4 variations.

All 28 patients with prolonged VKH had DR4. SSCP and RFLP analyses revealed that 26 patients had DRB1*0405 and 2 patients had DRB1*0410 (Table 1). In contrast, 7 of 18 patients (39%) with nonprolonged type had neither DRB1*0405 nor DRB1*0410. Three patients were DR4-negative. Four patients were DR4-positive yet DRB1*0405/0410-negative, with two having DRB1*0403 and two having DRB1*0406. Only 11 patients had either DRB1*0405 or DRB1*0410; nine patients had DRB1*0405, one had DRB1*0410, and one was a heterozygote of DRB1*0405 and DRB1*0410.

The frequency of DRB1*0405/0410 was higher in patients with prolonged VKH (28/28) than in patients with nonprolonged VKH (11/18). This difference was statistically highly significant by Fisher's exact test ($P = 0.00059$, $P_c = 0.0041$). A significant difference was also observed for the DRB1*0405 frequency (26/28 versus 10/18) ($P = 0.0044$, $P_c = 0.030$). Among 39 patients who had either DRB1*0405 or DRB1*0410, 28 (72%) had the prolonged type, whereas none of 7 patients without them had that type ($P = 0.00059$).

DR4 was found in 94 of 218 controls (43%). DRB1*0405 was the most frequent DR4 variant, followed by DRB1*0403, DRB1*0406, and DRB1*0410 (Table 1).

In the prolonged type, the relative risk for the combined specificity of DRB1*0405 and DRB1*0410 was 128, whereas it was 75 and 0 for DR4 and DRB1*0403/0406, respectively. In the nonprolonged type, the relative risk was highest for DR4 (6.6), and those for DRB1*0405/0410 and for DRB1*0403/0406 were 3.5 and 2.3, respectively.

**DISCUSSION.** This is the first study demonstrating significant differences in DR4 gene variants between the two clinical subgroups of VKH. All patients with the prolonged type had either DRB1*0405 or DRB1*0410, whereas 39% of the patients with the nonprolonged type had neither of them ($P = 0.00059$, $P_c = 0.0041$). DRB1*0405 frequency was also significantly higher in the prolonged type ($P = 0.0044$, $P_c = 0.030$).

In 1979, Mimura and colleagues proposed classifying VKH into two subgroups, prolonged and nonprolonged types. The nonprolonged type was defined as the type in which inflammation subsides within 6 months without recurrence, whereas the prolonged type was defined as the type in which inflammation does not subside after 6 months of proper treatment or it recurs. Mizote and colleagues later found that clinical features are significantly different between these subtypes. Nearly half of patients with prolonged VKH had the severe form of anterior uveitis; only 6% of patients with nonprolonged VKH had the severe form. Extraocular symptoms such as hearing loss, alopecia, poliosis, and vitiligo were more frequent among patients with the prolonged type. Reduction of visual acuity was observed only in patients with the prolonged type. The ratio of patients with the nonprolonged type to patients with the prolonged type was...
FIGURE 2. Representative gel of PCR-RFLP analysis. PCR-amplified DNA fragments from reference cells were digested with restriction endonucleases. Sac II digests DRB1*0401, 0402, 0404, 0405, and 0410, whereas Hinf I digests only DRB1*0406. Ava II digests all but DRB1*0402. Undigested DNA is 263 bp in size, and digested DNA is 205 bp, 173 bp, and 179 bp after Sac II, Hinf I, and Ava II digestion, respectively. We also used Hae II, Mnl I, and Hph I to assign samples.8 M, molecular weight marker (FX174/Hae III digest).

TABLE 1. DRB1*04 Variants in the Two Subtypes of VKH

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prolonged</td>
<td>Non-prolonged</td>
</tr>
<tr>
<td>DR4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0401</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0403</td>
<td>0</td>
<td>2 (11)</td>
</tr>
<tr>
<td>0404</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0405</td>
<td>26 (93†)</td>
<td>10 (56)</td>
</tr>
<tr>
<td>0406</td>
<td>0</td>
<td>2 (11)</td>
</tr>
<tr>
<td>0407</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0410</td>
<td>2 (7)</td>
<td>2 (11)</td>
</tr>
<tr>
<td>0405 or 0410</td>
<td>28 (100†)</td>
<td>11 (61)</td>
</tr>
</tbody>
</table>

* Includes two DRB1*0405/0410 and seven other DRB1*04/04 heterozygotes. We did not find DRB1*0405/0406 heterozygotes in our controls.
† Includes three DR4-negative patients and one DRB1*0405/0410 heterozygote.
‡ Significantly increased compared with controls at P < 1.0 × 10^-10.

approximately 1:2.2 Neither study investigated HLA, and this is the first study to investigate the immunogegetic background in these subtypes.

The sequences of DRB1*0405 and DRB1*0410 are identical except for amino acid 86: glycine in DRB1*0405 and valine in DRB1*0410.4 Three hyper-variable regions (amino acids 8–14, 26–37, and 67–74) that determine the HLA antigenicity and possibly most of the peptide-binding capacity of HLA proteins are identical between DRB1*0405 and DRB1*0410.4 Because of that, and because DRB1*0410 is the only DR4 genotype other than DRB1*0405 that was found in the prolonged type, we favor the hypothesis that DRB1*0410 plays a role similar to that of DRB1*0405 in determining the disease severity of VKH. Because DRB1*0410 did not increase statistically by itself, we could not prove this hypothesis. It should be noted, however, that there is no proof at present that DRB1*0405 and DRB1*0410 are immunologically different. They can be identical biologically, as are many other population-fixed polymorphisms.

DRB1*0405, DRB1*0410, and other DR4 variants observed in VKH, namely DRB1*0405 and
DRB1*0406, differ by two or three amino acids.\(^4\) Amino acid 74 is a nonpolar alanine in DRB1*0405 and DRB1*0410, but it is an acidic glutamate in DRB1*0403 and DRB1*0406. Amino acid 57 is a basic serine in DRB1*0405 and DRB1*0410, but it is an acidic aspartic acid in DRB1*0403 and DRB1*0406.\(^4\) In addition, DRB1*0406 has serine at amino acid 37, whereas DRB1*0405, DRB1*0410, and DRB1*0403 have tyrosine. These sites interact directly with antigenic peptides and thus affect the antigen presentation to T cells.\(^10\) We hypothesize that although all DR4 variants bind to a putative pathogenic peptide of VKH disease, the DRB1*0405/0410 variant is especially efficient in binding and presenting it to T cells because of their amino acid charges. Thus, it induces the severer form of the disease.

In patients with the nonprolonged type, the relative risk was higher for DR4 (6.6) than for its variants, DRB1*0405/0410 (3.5) or DRB1*0403/0406 (2.3). This suggests that DR4 specificity itself, rather than any variation, is the most important factor in the contraction of the nonprolonged type of VKH. Because DR4 specificity is determined primarily by the first and second hypervariable portions (amino acids 8–14 and 26–37) of DRB1 peptide,\(^4\) one attractive hypothesis is that these regions determine the susceptibility to VKH, whereas the third hypervariable portion and amino acid 57 determine the disease severity.

Because all the patients with the prolonged type had either DRB1*0405 or DRB1*0410, and patients without DR4, DRB1*0405, or DRB1*0410 did not develop the prolonged type, it is tempting to speculate that the DRB1*0405 and DRB1*0410 variant is a prerequisite factor to the development of the prolonged type. Even if this is the case, however, it is apparent that they are not sufficient. Nine patients were DRB1*0405- or DRB1*0410-positive, yet they did not contract the prolonged type. There must be other factors required in determining the disease severity of VKH.

In conclusion, this is the first study to compare genetic markers in two clinical subgroups of VKH. Because of the limited number of patients, this study should be considered preliminary. VKH is a relatively rare disease in Japan, and the nonprolonged type is in the minority.\(^1,2\) This study does, however, indicate that genetically determined factors affect the clinical course of VKH. We are currently investigating other genetic markers in VKH to elucidate what role they play in determining the severity of VKH.

**Key Words**
Vogt-Koyanagi-Harada disease, HLA-DR4, polymerase chain reaction, single-strand conformation polymorphism, restriction fragment length polymorphism

**References**


