Identification of Autoreactive T Cells in Vogt-Koyanagi-Harada Disease

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PURPOSE. To determine the finer specificity and immunologic features of autoreactive T cells in Vogt-Koyanagi-Harada (VKH) disease.

METHODS. T-cell clones (TCCs) specific to tyrosinase family proteins were raised from the peripheral blood mononuclear cells (PBMCs) of patients with VKH disease, and the response of the TCCs to 30-mer peptides was determined. The TCCs that were reactive to the peptides with strong binding sites for HLA DRB1*0405 were initially tested. Then, a finer specificity of these TCCs against 12- to 14-mer peptides was determined. The cytokine production of these clones was measured by ELISA.

RESULTS. A total of 62 stable TCCs were established from the PBMCs of five patients with VKH (28 clones against tyrosinase, 34 clones against tyrosinase-related protein [TRP]1). Five of 28 TCCs for tyrosinase and 2 of 34 for TRP1 were reactive to the 30-mer peptides with strong binding sites for HLA DRB1*0405. These seven clones showed proliferative responses to one or more of the 12- to 14-mer peptides that match the motif of the strong binding site for HDLDRB1*0405. Five of seven of the TCCs may be Th-helper (Th) type 1, one of the remaining TCCs may be Th0, and the other may be Th2.

CONCLUSIONS. The autoreactive T cells against tyrosinase and/or TRP1 may contribute to the development of VKH disease.


Vogt-Koyanagi-Harada (VKH) disease is an ocular inflammatory disease that manifests as aseptic meningitis with vitiligo and internal ear inflammation. The results of earlier studies indicate that VKH disease is probably a cell-mediated autoimmune disease against melanocytes. Thus, the lymphocytes of patients with VKH disease attach to and attack the melanocytes. The lymphocytes also proliferate when challenged by antigens from melanocytes or by crude soluble or insoluble fractions of melanocytes.

It has been established that VKH disease is highly related to HLA DRB1*0405 in Japanese and Asians. In our previous study, 9 of 10 patients had HLA DRB1*0405. Tyrosinase and tyrosinase-related protein (TRP1) are proteins of the tyrosinase gene family that are the enzymes involved in melanin formation and are expressed specifically in melanocytes. Functionally, tyrosinase catalyzes the hydroxylation of tyrosine to form dopa and the oxidation of dopa to dopaquinone. TRP1 is dihydroxyindole-2-carboxylic acid oxidase and converts dihydroxyindole-2-carboxylic acid to Eumelanin.

We have shown that tyrosinase family proteins can induce an autoimmune disease in rats that highly resembles human VKH disease, and the lymphocytes of patients with VKH disease are reactive against the peptides derived from tyrosinase family proteins. The lymphocytes from patients with VKH showed proliferative responses to one or several peptide groups containing HLA DRB1*0405 binding sites.

Liu et al. reported that the induction of experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (MS) in mouse, is related to the stability of the antigenic peptide and major histocompatibility molecule complex. The intermediate-to-weak, but not the strong, binder peptide is pathogenic, because the T cells against the intermediate-to-weak binders may escape immune tolerance. On the contrary, in the EAE induced by the proteolipid protein, the strong binder peptide (131-151) induces the EAE. Other experimental models using transgenes show that the predominant pathogenic T cells in EAE or in nonobese diabetic (NOD) mice, a model of human diabetes mellitus, recognize the strong binders. In human MS, approximately 65% of the TCCs established from patients recognize a subimmunodominant site of human myelin basic protein (MBP; 111-129) that binds weakly to DRB1*0401 and has a limited heterogeneity of rearrangement of T-cell receptors. More recently, it was reported that in human MS, autoreactive T cells against immunodominant sites of MBP are mostly CD4+ and CD45RA- cells, and these cells may be pathogenic.

The conclusions made from the results of experimental models and human data have some discrepancies, and it is important to know the character and target peptides of autoreactive T cells in VKH disease. To clarify the role of the autoreactive T cells in patients with VKH disease, we established TCCs specific to tyrosinase family proteins (tyrosinase and TRP1) from the peripheral blood of patients. We investigated the responses of the TCCs to synthesized 30-mer peptides and then identified the specificity of the selected TCCs that have the potential for a pathogenic effect. We refined the specificity by testing the TCCs against 12- to 14-mer peptides derived from tyrosinase and TRP1 and also examined the character of these selected TCCs.

**MATERIALS AND METHODS**

**Patients**

We studied patients with VKH disease at the Akita University School of Medicine Hospital, who were in the initial, untreated stage (Table 1). The diagnosis was made according to the guidelines of the Uveitis Society of Japan with examination of the fundus and fluorescein angiography. The subjects were one man and four women with a mean age of 44.4 years (range, 37–49). Cerebrospinal fluid and genotype of the HLA were used for diagnostic confirmation.

After informing each patient of the purpose of this study, consent was obtained. The research conformed to the recommendations of the Declaration of Helsinki.
Genotyping of the HLA DR Region

HLA-DRB1 alleles were determined by polymerase chain reaction with the restriction fragment length polymorphism (PCR-RFLP) method. The HLA-DR alleles were designated according to the World Health Organization Nomenclature Committee for factors of the HLA system.

Peptides

A panel of 30-mer peptides that overlapped by nine amino acids was synthesized by the 9-fluorenymethyl carbonyl (Fmoc) solid-phase method to cover the entire human tyrosinase and TRP1 sequence. Twenty-five peptides for tyrosinase and 22 peptides for TRP1 were synthesized (Table 2). Each peptide mixture was used to establish the TCCs as the stimulating antigen.

A panel of 12- to 14-mer peptides that overlapped by nine amino acids was also synthesized by the Fmoc solid-phase method. Eighteen peptides for tyrosinase position 82-146 (TYRO 46-1 to -18), 18 peptides for tyrosinase position 414-476 (TYRO 2024-1 to -18), and 28 peptides for TRP1 position 201-294 (TRP1 912-1 to -28) were synthesized (Table 3). These peptides covered the regions that had induced proliferative responses of the lymphocytes from patients with VKH.

Establishment of TCCs

TCCs were raised by the limiting-dilution method from the PBMCs of five patients with VKH disease. Briefly, lymphocytes were separated from the peripheral blood of the patients by a kit (Lymphoprep; Nycomed, Oslo, Norway). Two milliliters of PBMCs (1.0 × 10⁶) in RPMI 1640 (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum and 5% T-cell growth factor (TCGF) were stimulated with a mixture of 30-mer peptides of tyrosinase or TRP1. Each peptide was used at a final concentration of 10 μM. Lymphocytes were restimulated weekly under the same conditions except for the addition of 10% of culture medium or phytohemagglutinin (PHA; Difco, Detroit, MI) and APCs were added. After 72 hours of culture, 1 μg (10 μl/well) of [³H]thymidine (Amersham, Buckinghamshire, UK) was added. After 24 hours of incubation, the lymphocytes were harvested, and the uptake of the [³H]thymidine was measured. A stimulation index of 3.0 or more was considered significant.

Cytokine Assay and Characterization of TCCs

The cell-free samples for the cytokine assay were obtained by centrifugation of the culture media from the 24-well plates. They were collected after 2 days of restimulation with fresh medium, irradiated APCs, and 50 μg/ml of the stimulating antigens. The TCCs were cultured with stimulation of the antigens and irradiated APCs without TCGF and/or IL-2 for 7 days before the proliferation assay.

ELISA kits for IL-2, IL-4, and IFN-γ (Genzyme, Cambridge, MA) were used to detect cytokines. The sensitivity of each assay kit was as follows: IL-2, 4.0 pg/ml; IL-4, 0.6 pg/ml; and IFN-γ, 3.0 pg/ml.

The surface markers of the TCC were determined by an immunostaining method, in which the cells were smeared and stained by anti-CD3, anti-CD4, and/or anti-CD8 antibodies.

RESULTS

Genotype of the HLA DR Region in VKH Disease

All the patients with VKH disease had HLA DRB1*0405 (Table 1).

Establishment of TCCs

Twenty-eight tyrosinase-specific and 3i TRP1-specific TCCs were established from the peripheral blood of patients with VKH disease. Eight clones from patient 1 (4 against tyrosinase, 4 against TRP1), 9 from patient 2 (6 against tyrosinase, 3 against TRP1), 15 from patient 3 (8 against tyrosinase, 7 against TRP1).

T-Cell Proliferation Assay

Proliferation assays against the peptides of tyrosinase or TRP1 were performed in triplicate with a modified method as shown in Reference 23. Briefly, 2 × 10⁶ T cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. The peptide (50 μg/200-μl well) and APCs were added to the medium. For control cultures, the same amount of culture medium or phytohemagglutinin (PHA; Difco, Detroit, MI) and APCs were added. After 72 hours of culture, 1 μCi (10 μCi/ml) of [³H]thymidine (Amersham, Buckinghamshire, UK) was added. After 24 hours of incubation, the lymphocytes were harvested, and the uptake of the [³H]thymidine was measured. A stimulation index of 3.0 or more was considered significant.

TCCs in Vogt-Koyanagi-Harada Disease 2005

Table 1. Patients With VKH Disease Included in the Study

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Table 2. The 30-Mer Peptides Derived from Tyrosinase and TRP1

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TYR-26 474-499 is a membrane-spanning portion. The positions 132-141, 233-242, 248-437, and 447-456 of tyrosinase are putative strong binding sites to HLA-DRB1*0405. The position 1-24 of TRP1 is a signal peptide and the position 481-501 is membrane-spanning portion. The positions 246-255, 294-303, and 398-407 of TRP1 are putative strong binding sites to HLA-DRB1*0405.
TRP1, 14 from patient 4 (4 against tyrosinase, 10 against TRP1), and 16 from patient 5 (6 against tyrosinase, 10 against TRP1) were established (Table 4).

We investigated the responses of the TCCs against each 30-mer peptide of tyrosinase positions 82-146 and 414-476 and TRP1 position, 201-294 because a positive reaction of the lymphocytes from patients with VKH disease had been detected against these positions previously. In addition, the peptides covering these positions have relatively strong binding sites for HLA DRB1*0405, and these sites may be a target of functional pathogenic autoreactive T cells.

Five tyrosinase-specific TCCs were designated SS-F12 (patient 1), NK-1E4 (patient 2), MS-1F6 (patient 3), KE-2G2 (patient 4), and EK-1C1 (patient 5). These TCCs responded to the synthesized 12- to 14-mer peptides (Fig. 1). For the TRP1-specific TCCs, both TCC NK-2B8 and TCC KE-1E7 showed proliferative responses to the synthesized 12- to 14-mer peptides (Table 4).

All TCCs thus generated were CD4+ and anti-CD8+ by staining with anti-CD4+ and anti-CD8+ antibodies (data not shown).

Cytokine Production by TCCs

Cytokine production was analyzed for the seven TCCs (five tyrosinase-specific TCCs and two TRP1-specific TCCs); the results are shown in Table 5. All the clones except MS-1F6 showed higher levels of IFN-γ production. Three of five tyrosinase-specific TCCs, SS-F12, NK-1E4, and KE-2G2, did not produce detectable levels of IL-4, but two of the TCCs, MS-1F6 and KE-1C1, produced 18 pg/ml and 16.4 pg/ml IL-4, respectively. TRP1-specific TCCs, NK-2B8 and KE-1E7, produced higher levels of IFN-γ, but IL-4 was not detectable.

Fine Specificity of TCCs

We investigated the proliferative responses of the seven TCCs against the synthesized 12- to 14-mer peptides (Fig. 1). For the tyrosinase-specific TCCs, SS-F12 proliferated against the TYRO 46-17 (position [p]131-143) and TYRO 46-18 (p134-146), TCC NK-1E4 showed proliferative responses to both TYRO 2024-4 (p423-434) and 2024-5 (p426-437), MS-1F6 and KE-1C1 proliferated against TYRO 2024-5 (p426-437), and KE-2G2 responded to TYRO 2024-4 (p423-434), 2024-5 (p426-437), and 2024-6 (p429-440). The stimulation index of KE-2G2 was approximately the same for these peptides.

For the TRP1-specific TCCs, both TCC NK-2B8 and TCC KE-1E7 responded to TRP1 912-16 (p246-257). The TCC, KE-1E7 showed proliferative responses to TRP1 912-15 (p243-254) and 912-17 (p249-260; Table 3).

The amino acid sequence of the panels of 12- to 14-mer peptides used for fine specificity of TCCs. The peptides were 12-14 mer and were synthesized stepwise by three amino acids to cover the regions, tyrosinase 82-146, tyrosinase 414-476, and TRP1 201-294.
DISCUSSION

In the induction of tissue-specific autoimmune diseases, autoreactive T cells play a critical role. To determine the specificity and characteristics of the autoreactive T cells involved in VKH disease, we established 62 TCCs from the tyrosinase- or TRP1-reactive PBMCs of five patients with VKH disease. There was not much variation in the number of the TCCs established for each protein and for each person.

We selected the TCCs that were reactive to tyrosinase positions 82-146 (TYR-4 to -6) and 414-476 (TYR-20 to -24) or to TRP1 position 201-294 (TRP1-9 to -12) because the lymphocytes of patients with VKH disease had shown a positive reaction against these positions (Table 2). The positions of the peptides may have immunodominant (relatively strong binding) sites for HLA DRB1*0405. In addition, these sites may be targets of functional pathogenic autoreactive T cells. We then determined the fine specificity and cytokine production profile of these TCCs.

It is well known that VKH disease is highly correlated with HLA DRB1*0405 (or 0410). In our study, all the patients with VKH had HLA DRB1*0405. The amino acids sequences that bind to HLA DRB1*0405 have been well-studied. Based on these reports, tyrosinase has four binding sites for HLA DRB1*0405 (positions 132-141, 233-242, 428-437, and 447-506), and TRP1 has three binding sites (positions 246-255, 294-303, and 398-407).

Seven TCCs, five for tyrosinase and two for TRP1, were selected. These clones were obtained approximately evenly from all the patients. The TCCs, NK-1E4, MS-1F6, KE-2G2 and EK-1C1, were reactive to TYRO 2024-5, MYPFIPILYRNGD (p426-437). The TCC SS-F12 showed reactivity to both TYRO 46-17 and 46-18. This clone may be reactive to the common sequence FFAYILTIKHK (p134-143) of TYRO 46-17 and 46-18. The TCC NK-1E4 showed proliferative responses to TYRO 2024-4 and 2024-5. This clone may be reactive to the common sequence MYPFIPILYG (p426-434) of TYRO 2024-4 and 2024-5. The TCC KE-2G2 responded to TYRO 2024-4, TYRO 2024-5, and TYRO 2024-6. For these peptides, the common sequence is PFLYR (p429-434). KE-2G2 may be the core sequence required for proliferation. These sequences matched the putative strong binding sites for HLA DRB1*0405 except for the anchor residues of the third pocket. There were two anchor residues for the first and second pockets of the HLA molecule, but there was no third anchor residue.

For the TCCs against TRP1, NK-2B8 was reactive to TRP1 912-16, LPWYWFATGKKNV (p246-257). This sequence completely matched the putative strong binding site of the HLA DRB1*0405 molecule. The TCC KE-1C1 was reactive to TRP1 912-15, 912-16, and 912-17. The common sequence, WNFATG (p249-254), may be the core sequence necessary for proliferation. This sequence had the second and third anchor residues but not the first anchor residue for the pockets of the HLA DRB1*0405 molecule. All these peptides and HLA molecules may form a relatively stable complex. All seven TCCs were reactive against the tyrosinase positions 82-146 and 414-476 or TRP1 position 201-294, which are relatively strong binding sites for HLA DRB1*0405. The stimulation index of these TCCs was not as high as that reported in other studies, because the thymidine incorporation of unstimulated TCCs was two to three times, or even more, higher than in other reports. Thus, the higher counts in the unstimulated control may be the reason the stimulation index was not as high.

Although the reactive sites for the other TCCs were not confirmed by testing fine specificity, they were weakly reactive to diverse kinds of 30-mer peptides covering entire tyrosinase or TRP1 (stimulation index <3.0; data not shown). There were only seven TCCs reactive to these strong binding sites, but the ratio of the TCCs—for example, the TCCs reactive against tyrosinase position 426-437 (TYRO 2024-5)—was 4:28. This ratio may be higher than that of other sites. Although we cannot make a definitive conclusion from these results, because the total number of the TCCs was small, the results matched, to some extent, the recent results in patients with MS. TCCs reactive to MBP were established separately from naïve T cells (CD45RA+) or from memory T cells (CD45RO+) of PBMCs of patients with MS. Most of the TCCs from naive T cells were established against the immunodominant sites and those from memory T cells were established against the other sites diversely. Most of these TCCs from CD45RA naïve T cells were Th helper (Th) type 1. It has been proposed that these T-cell subsets may be the source of potentially pathogenic effector CD4+ T-cell responses in MS.

It has been reported that T-cell lines from patients with VKH disease are reactive against tyrosinase position 188-208, which has the first and third anchor residues, but does not have the second anchor residue for DRB1*0405 (p188-208). In normal volunteers who had HLA DR4, none of the T-cell lines was reactive against any peptides derived from tyrosinase. In our experiment, we could not establish TCCs against this site. It is difficult to compare these results directly with our results, because we established TCCs against a tyrosinase peptide mixture and identified the fine specificity. However, Kobayashi et al. used T-cell lines for determining fine specificity. This difference may be due to the differences in experimental procedures and/or the differences in the patients used in the experiments. In both experiments and other reports on patients with MS, autoreactive T cells against candidate antigens were established, not only against immunodominant sites but also against many other sites of the antigens.

In the present study, the TCCs for the immunodominant sites of tyrosinase were reactive to different immunodominant sites in the individual patients. The TCC SS-F12 from patient 1 was raised against tyrosinase position 134-143, but the other clones—NK-1E4 from patient 2, MS-1F6 from patient 3, KE-2G2 from patient 4, and EK-1C1 from patient 5—were raised against position 420-437.
FIGURE 1. Fine specificity of the TCCs. The T-cell proliferation assay against the 12- to 14-mer peptides was performed in triplicate. The numbers on the vertical axis are the peptide numbers, and the numbers on the horizontal axis are the stimulation index (S. I.). A stimulation index higher than 3.0 was considered to be significant. (A) TCC SS-F12 from patient 1, (B) TCC NK-1E4 from patient 2, (C) TCC MS-1F6 from patient 3, (D) TCC KE-2G2 from patient 4, (E) TCC EK-1C1 from patient 5, (F) TCC NK-2B8 from patient 2, and (G) TCC KE-1E7 from patient 4.
The TCCs SS-F12, NK-1E4, KE-2G2, and EK-1C1 against tyrosinase and NK-2B8 and KE-1E7 against TRP1 produced significantly higher levels of IFN-γ. The TCC MS-1F6 produced mildly higher levels of IL-4 and IL-2. But the production of IFN-γ was not detected in this assay system. The cytokine-producing profiles of the TCCs showed that five of seven TCCs may be Th1, TCC EK-1G1 may be Th0, and TCC MS-1F6 may be Th2. These results suggest that these TCCs may contribute to the immune reaction in VKH disease. The role of Th1 T lymphocytes in autoimmune diseases is supported by other reports. In human MS, TCCs specific for immunodominant MBP peptides are predominantly Th1 or Th0.25 In VKH disease, although the Th1 cytotoxic cells from the patients were not established as TCCs, the cytotoxic cell lines against melanocytes are Th1 cells and may be pathogenic to melanocytes.32

These observations suggest that TCCs reactive to the relatively strong binding sites of tyrosinase family proteins may play an important role in the induction of VKH disease; however, we do not know the exact role played by these T cells. To answer this question, it is important to know the functional avidity of these TCCs and peptide–HLA complex. We are now investigating the affinity of T cell receptors of these clones and peptide–HLA complex.

References