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HTLV-1 bZIP Factor–Specific CD4 T Cell Responses in Adult T Cell Leukemia/Lymphoma Patients after Allogeneic Hematopoietic Stem Cell Transplantation

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We document human T lymphotropic virus type 1 (HTLV-1) bZIP factor (HBZ)-specific CD4 T cell responses in an adult T cell leukemia/lymphoma (ATL) patient after allogeneic hematopoietic stem cell transplantation (HCT) and identified a novel HLA-DRB1*15:01–restricted HBZ-derived naturally presented minimum epitope sequence, RRAEKKAADVA (HBZ114–125). This peptide was also presented on HLA-DRB1*15:02, recognized by CD4 T cells. Notably, HBZ-specific CD4 T cell responses were only observed in ATL patients after allogeneic HCT (4 of 9 patients) and not in nontransplanted ATL patients (0 of 10 patients) or in asymptomatic HTLV-1 carriers (0 of 10 carriers). In addition, in one acute-type patient, HBZ-specific CD4 T cell responses were absent in complete remission before HCT, but they became detectable after allogeneic HCT. We surmise that HTLV-1 transmission from mothers to infants through breast milk in early life induces tolerance to HBZ and results in insufficient HBZ-specific T cell responses in HTLV-1 asymptomatic carriers or ATL patients. In contrast, after allogeneic HCT, the reconstituted immune system from donor-derived cells can recognize virus protein HBZ as foreign, and HBZ-specific immune responses are provoked that contribute to the graft-versus-HTLV-1 effect.

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Abbreviations used in this article: AC, asymptomatic carrier; ATL, adult T cell leukemia/lymphoma; CR, complete remission; HAM, human T lymphotropic virus type 1–associated myelopathy; HBZ, human T lymphotropic virus type 1 bZIP factor; HCT, hematopoietic stem cell transplantation; HTLV-1, human T lymphotropic virus type 1.
Materials and Methods

Primary human cells

Blood samples were obtained from healthy volunteers, HTLV-1 asymptomatic carriers (ACs), and ATL patients. Mononuclear cells were isolated with Ficoll-Paque (Pharmacia, Peapack, NJ). Genotyping of HLA-DR, HLA-DQ, and HLA-DP was performed using an WAKFlow HLA-typing kit (WAKUNAGA Pharmacy, Hiroshima, Japan). Diagnosis and classification of clinical subtypes of ATL were according to the criteria proposed by the Japan Lymphoma Study Group (32). All donors provided informed written consent before sampling, according to the Declaration of Helsinki, and the current study was approved by the institutional ethics committees of Nagoya City University Graduate School of Medical Sciences.

Cell lines

ATN-1, MT-1, TL-Om1, and ATL102 are ATL cell lines; MT-2, MT-4, and TL-Su are HTLV-1-immortalized lines; and K562 is a chronic myelogenous leukemia blast crisis cell line (8, 33). Genotyping of HLA-DR, HLA-DQ, and HLA-DP was performed using a WAKFlow HLA-typing kit.

Expansion of HBZ-specific T cells

PBMCs from ATL patients or HTLV-1 ACs were suspended in RPMI 1640 (Cell Science and Technology Institute, Sendai, Japan) supplemented with 10% human serum and 10 μM synthetic HBZ-derived peptides at a cell concentration of 2 × 10^6/ml. The peptides were purchased from Invitrogen (Carlsbad, CA). The cell suspension (2 × 10^6 cells) was cultured at 37°C in 5% CO2 for 2 d, and an equal volume of RPMI 1640 supplemented with 100 IU/ml IL-2 was added. After subsequent culture for 5 d, an equal volume of ALyS505N (Cell Science and Technology Institute) supplemented with 100 IU/ml IL-2 was added, and the cells were cultured with appropriate medium (ALyS505N with 100 IU/ml IL-2) for an additional 7 d.

Abs and flow cytometry

PerCP-conjugated anti-CD8 mAb (SK1; EbiScience, San Diego, CA) and PE-conjugated anti-CD4 mAb (SFC124T4D11T; Beckman Coulter, Fullerton, CA) were used. For assessing HLA class II expression, PE-conjugated anti–HLA-DR (G566-60; BD Biosciences, San Jose, CA), anti–HLA-DQ (HLA-DQ1; BioLegend, San Diego, CA), or appropriate isotype-control mAbs were used. For intracellular IFN-γ and TNF-α staining, the expanded cells were cocultured with or without target cells or synthetic peptides at 37°C in 5% CO2 for 3 h, after which brefeldin A (BD Biosciences) was added at 2 μg/ml. The cells were then incubated for an additional 2 h. Subsequently, they were fixed in 10% formaldehyde and stained with FITC-conjugated anti–TNF-α (45.15; Beckman Coulter) or allophycocyanin-conjugated anti–TNF-α (MAb11; EbiScience) mAbs with 0.25% saponin for 60 min at room temperature. To determine HLA restriction, HLA-blocking experiments were conducted. The expanded cells were preincubated with 20 μg/ml anti–HLA-DR (L243; BioLegend), 20 μg/ml anti–HLA-DQ (ISPV1L3; Beckman Coulter), or appropriate isotype control mAbs (20 μg/ml) at 37°C in 5% CO2 for 1 h, after which they were stimulated with the peptide or the cell lines (ATN-1 and K562). Cells were analyzed on a FACScalibur (BD Biosciences) with the aid of FlowJo software (Tree Star, Ashland, OR).

Quantitative RT-PCR

Total RNA was isolated with RNeasy Mini Kits (QIAGEN, Tokyo, Japan). Reverse transcription from the RNA to first-strand cDNA was carried out using High Capacity RNA-to-cDNA Kits (Applied Biosystems, Foster City, CA). HBZ and ß-actin mRNA were amplified using TaqMan Gene Expression Assays with the aid of an Applied Biosystems StepOnePlus. The primer set for HBZ was as follows: sense, 5'-TCGACCTGAGCTTATGAACCTACCTAGA-3' and antisense, 5'-GACACAGGCAAGCATCGA-A-3'. All values given are means of triplicate determinations.

Results

T cell responses against synthetic peptides overlapping by 10 aa and covering the entire sequence of the spliced HBZ protein

Because it was reported that HTLV-1 Tax-specific T cells were induced in some ATL patients after allogeneic HCT (10, 11), we initially tried to expand HBZ-specific T cells using PBMCs from an ATL patient who received allogeneic HCT with reduced-intensity conditioning and has been in complete remission (CR) for >3 y (patient #1 after HCT). PBMCs were stimulated with a mixture of 1 16-mer and 19 20-mer synthetic peptides overlapping by 10 aa and covering the entire sequence of the spliced HBZ protein (peptides number 1–20, Fig. 1), at a concentration of 10 μM each. The expanded cells were analyzed by forward scatter height and side scatter height levels, and the lymphocyte population was determined and plotted to show CD4 and CD8 positivity (Fig. 2A, left panels). The expanded CD8 T cells responded weakly to stimulation with these 20 overlapping peptides relative to controls without peptide stimulation, as assessed by IFN-γ production (Fig. 2A, upper middle panels) but not TNF-α (Fig. 2A, lower middle panels). In contrast, the expanded CD4 T cells responded to stimulation by the 20 overlapping peptides by producing both IFN-γ (Fig. 2A, upper right panels) and TNF-α (Fig. 2A, lower right panels). Because the response of the stimulated and expanded CD4 T cells was stronger than the CD8 response, we focused on the CD4 T cell response against HBZ in patient #1 after HCT.

PBMCs from this patient (#1 after HCT) were stimulated with a mixture of five overlapping peptides consisting of peptides 1–4, 5–8, 9–12, 13–16, and 17–20 (Fig. 1). The expanded CD4 T cells responded to the peptide mixture 9–12 better than to control (no peptides). They produced both IFN-γ (Fig. 2B, upper panels) and TNF-α (Fig. 2B, lower panels). The expanded CD4 T cells responded very weakly to the peptide mixtures 13–16 and 17–20 by producing TNF-α but not IFN-γ. No responses were observed against the peptide mixtures 1–4 or 5–8 (Fig. 2B). These data indicate that the epitope of HBZ recognized by CD4 T cells from the patient was present in peptides 9–12, within HBZ aa residues 81–130 (Fig. 1).

Next, PBMCs from the same patient were stimulated with four synthetic peptides: 9, 10, 11, and 12. The expanded CD4 T cells responded to peptide 12 by producing both IFN-γ (Fig. 2C, upper panels) and TNF-α (Fig. 2C, lower panels). The cells did not respond significantly to the other peptides (9, 10, or 11). These results narrow down the specific epitope of HBZ recognized by the CD4 T cells from the patient to a sequence within peptide 12: HBZ aa 111–130 (Fig. 1).

Determination of the minimum epitope sequence of HBZ recognized by CD4 T cells

Seven synthetic peptides (12-1, 12-2, 12-3, 12-4, 12-5, 12-6, 12-7) representing parts of peptide 12 were prepared (Fig. 3A). Responses of the CD4 T cells, which had been stimulated by peptide 12, to these different peptides were tested. The expanded CD4 T cells responded better to peptides 12, 12-1, 12-2, 12-3, and 12-4 for >3 y (patient #1 after HCT). PBMCs were stimulated with a mixture of 1 16-mer and 19 20-mer synthetic peptides overlapping by 10 aa and covering the entire sequence of the spliced HBZ protein.
by producing both IFN-γ and TNF-α. These cells did not respond to peptides 12-5, 12-6, or 12-7 (Fig. 3B). These data indicate that the N terminus of the minimum epitope sequence of HBZ recognized by the CD4 T cells from the patient is arginine, located at HBZ114 (Fig. 3A). Because the expanded CD4 T cells responded to peptide 12-4, the C terminus of the minimum epitope sequence of HBZ must be inside of alanine, located at HBZ125.

Next, three synthetic peptides (12-4-1, 12-4-2, 12-4-3; sequences were HBZ114–124, HBZ114–123, and HBZ114–122, respectively) were prepared to determine the C terminus of the minimum epitope sequence of HBZ (Fig. 3C). The expanded CD4 T cells responded to peptides 12-1 and 12-4 (positive controls) but not to 12-4-1, 12-4-2, 12-4-3, or a negative control peptide 12-7 (Fig. 3D). These data demonstrate that the minimum epitope sequence of HBZ recognized by the CD4 T cells from the patient was RRRAEKKAADV A (HBZ114–125).

**Determination of the HLA allele on which the identified HBZ-derived peptides are presented to CD4 T cells**

We investigated whether HBZ-specific CD4 T cells also recognized naturally processed and presented peptides. Thus, we initially determined HBZ expression by ATL or HTLV-1–immortalized cell lines and found that it was expressed by all of the lines tested (ATN-1, MT-1, MT-2, MT-4, TL-Su, TL-Om1, ATL102), regardless of their Tax mRNA expression (Fig. 4A, below the graph). HBZ expression levels of these established lines were almost as high as those of PBMCs containing >50% ATL cells obtained from 12 patients with the acute or chronic type of disease. K562 did not express HBZ, as might be expected, and all primary ATL cells tested were HBZ+, consistent with an earlier study (Fig. 4A) (25). Next, we assessed the expression of HLA class II by the cell lines. The ATL or HTLV-1–immortalized cell lines tested were all positive for both HLA-DR and HLA-DQ (Fig. 4B). These observations indicate that ATN-1, MT-1, MT-2, MT-4, TL-Su, TL-Om1, and ATL102 had the potential to present the HBZ-derived peptides on their HLA-DR or HLA-DQ molecules.

Next, we examined the responses of HBZ-specific CD4 T cells from patient #1 after HCT against K562 or HBZ-expressing lines of different HLA types. The responses of HBZ-specific CD4 T cells to the lines were evaluated without the addition of peptide. The CD4 T cells that had been expanded from patient #1 after HCT using peptide 12 responded to peptide 12-1 (positive control) but not to K562, which expressed no HBZ (negative control) (Fig. 4C, upper six panels). When tested against ATL or HTLV-1–immortalized cell lines, the CD4 T cells responded strongly to ATN-1 and TL-Su (Fig. 4C, lower panels). Comparing the HLA class II types of the donor of the effector CD4 T cells (patient #1 after HCT) with ATN-1 and TL-Su showed that HLA-DRB1*15:01 and

**FIGURE 2.** T cell responses against synthetic peptides overlapping by 10 aa and covering the entire sequence of the spliced HBZ protein. (A) PBMCs from patient #1 after HCT were expanded by stimulating with a mixture of 19 20-mer and 1 16-mer synthetic peptides overlapping by 10 aa and covering the entire sequence of the spliced HBZ protein. The responses of expanded CD8 and CD4 T cells to each of the overlapping peptides were evaluated by the production of IFN-γ or TNF-α. The percentage of responding cells in the upper gate (CD8+ or CD4+ and IFN-γ+ or TNF-α+ cells) relative to the cells in the lower gate (CD8+ or CD4+ and IFN-γ− or TNF-α− cells) is indicated in each flow cytometry panel. (B) PBMCs from patient #1 after HCT were expanded by stimulating with five overlapping peptide mixtures consisting of peptides 1–4, 5–8, 9–12, 13–16, and 17–20. (C) PBMCs from patient #1 after HCT were expanded by stimulating with four synthetic peptides: 9, 10, 11, and 12. The responses of expanded CD4 T cells to each synthetic peptide were evaluated by the production of IFN-γ or TNF-α. The percentage of responding cells in the upper gate relative to the cells in the lower gate is indicated in each flow cytometry panel. Each result is representative of three independent experiments.
HLA-DQB1*06:02 were shared by all three (Table I). In addition, the CD4 T cells responded to MT-2, TL-Om1, and ATL102 to a lesser degree (Fig. 4C, lower panels); these three lines were found to share HLA-DRB1*15:02 and HLA-DQB1*06:01 (Table I). Together, these results indicate that the HBZ-specific CD4 T cell responses from patient #1 after HCT were restricted by HLA-DRB1*15:01 or HLA-DQB1*06:02, as well as by HLA-DRB1*15:02 or HLA-DQB1*06:01. In contrast, the peptide-sensitized CD4 T cells did not respond to MT-1 or MT-4 (Fig. 4C, lower panels), consistent with the present observations that the epitope of HBZ recognized by such CD4 T cells was restricted by HLA-DRB1*15:01/HLA-DQB1*06:02 and HLA-DRB1*15:02/HLA-DQB1*06:01.

Next, we tested whether HLA-DR or HLA-DQ restricted the presentation of the HBZ-derived peptide. CD4 T cells expanded by peptide 12 no longer responded to specific stimulation by peptide 12 in the presence of anti–HLA-DR–blocking mAb by producing IFN-γ (Fig. 5A, lower left panels); these results indicate that the HBZ-specific CD4 T cells responded to specific stimulation by peptide 12 in the presence of anti–HLA-DR–blocking mAb (Fig. 5A, upper left panels). The CD4 T cells also still responded to peptide 12 in the presence of anti–HLA-DQ–blocking mAb (Fig. 5A, lower right panels) and its isotype mAb (Fig. 5A, upper right panels). These observations from Ab-blocking experiments, together with the results shown in Fig. 4, indicate that the epitope sequence of HBZ recognized by the CD4 T cells from patient #1 after HCT were restricted by HLA-DR, specifically HLA-DRB1*15:01 and HLA-DRB1*15:02.

Clinical significance of the specific CD4 T cell response against HBZ

The data presented thus far pertained to CD4 T cells obtained from only one patient (patient #1 after HCT). Therefore, we used HBZ peptide 12 to stimulate and expand 28 PBMC samples obtained from 27 other HTLV-1–infected individuals who carried HLA-DRB1*15:01 or HLA-DRB1*15:02. PBMCs were obtained from 10 HTLV-1 ACs, 10 ATL patients who had not undergone allogeneic HCT, and 8 ATL patients after allogeneic HCT. Among them, PBMCs from one individual (patient #2) were tested at different disease stages (i.e., CRs before and after allogeneic HCT). HBZ-specific CD4 T cell responses were absent in all 10

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HTLV-1 ACs, as well as in all 10 nontransplanted ATL patients (of whom 9 were in CR after systemic chemotherapy and the other was of smoldering type under observation only). In contrast, specific CD4 T cell responses to HBZ were observed in three of the eight additional ATL patients who were in CR after allogeneic HCT (patients #2, #3, and #4). The CD4 T cells from patient #2 and #4 after HCT responded to HBZ peptide 12 by producing both IFN-γ and TNF-α (Fig. 6, right panels). In patient #3, no TNF-α response was observed, but there was a clear IFN-γ response to HBZ peptide 12 (Fig. 6, lower left panels). Thus, specific CD4 T cell responses against HBZ were observed in four of nine recipients after allogeneic HCT (44%) but in no other ATL patients. Among the patients examined in this study, one patient with acute-type ATL received systemic chemotherapy and achieved CR. Subsequently, she received allogeneic HCT from an HLA-A, B, DR-matched HTLV-1 noninfected sibling donor and maintained CR (patient #2 after HCT). Although HBZ-specific CD4 T cell responses were not present at CR before allogeneic HCT in this patient (Fig. 6, upper left panels), they developed after transplantation (Fig. 6, upper right panels).
Discussion

In the current study, we demonstrated the presence of HBZ-specific CD4 T cells in an ATL patient after allogeneic HCT and determined the minimum sequence of a novel HLA-DRB1*15:01–restricted HBZ-derived epitope to be RRRAEKKAADV A (HBZ114–125). HBZ peptides including the sequence HBZ114–125 were also presented on HLA-DRB1*15:02 and recognized by CD4 T cells. To the best of our knowledge, this is the first report to identify naturally processed and presented HLA-DR–restricted epitopes derived from HBZ on the surface of ATL cells. In an earlier study, an HBZ peptide–specific CTL line was established from an HLA-A*02:01+ individual, using peptides derived from the HBZ sequence. The peptides were selected by computer algorithms available at the BioInformatics and Molecular Analysis Section Web site (http://www-bimas.cit.nih.gov/molbio/hla_bind/) and the SYFPEITHI Web site (http://www.syfpeithi.de/) for strong binding affinity to the HLA-A*02:01 molecule. However, the established CTL line recognized the corresponding peptide-pulsed
HLA-A*02:01+ cells but not ATL cells (29). Therefore, it was not determined whether HBZ-derived peptides could be naturally presented on cells from HTLV-1–infected people. Another earlier study (30) demonstrated that HBZ expression was a critical determinant of viral persistence in the chronic phase of HTLV-1 infection. That novel study was performed using experimentally validated epitope-prediction software (34), but it did not determine the HBZ-derived epitope sequence or the corresponding HLA allele presenting it.

In the current study, no HLA-DRB1*15:01–restricted or HLA-DRB1*15:02–restricted HBZ-specific CD4 T cell response was observed in any ATL patients who had not undergone allogeneic HCT or in any HTLV-1 ACs. We surmise that HTLV-1 transmission from mothers to infants through breast milk in early life induces tolerance to HBZ, but not to Tax, by unknown mechanisms, resulting in insufficient HBZ-specific T cell responses in HTLV-1–infected individuals. This would be consistent with the persistent expression of HBZ in HTLV-1–infected cells (2, 25). In contrast, the finding that HLA-DRB1*15:01–restricted or HLA-DRB1*15:02–restricted HBZ-specific CD4 T cell responses were detected in ATL patients after allogeneic HCT requires explanation. Our hypothesis is that, after allogeneic HCT, the reconstituted immune system from donor-derived hematopoietic stem cells can recognize virus protein HBZ as foreign, although its expression is low, and HBZ-specific immune responses are provoked because of the lack of tolerance induction under these circumstances. In one patient with acute-type disease, HBZ-specific CD4 T cell responses were not observed in PBMCs at the time of CR before HCT, but they became detectable after allogeneic HCT. This observation supports our hypothesis. An earlier study (36) reported that HBZ-specific T cell responses were detected in some patients with HTLV-1–associated myelopathy (HAM). Unlike ATL, HAM can occur in individuals infected with HTLV-1 by any route of transmission, such as sexual intercourse (37). Therefore, some patients with HAM, infected with HTLV-1 after reaching adulthood (i.e., who became infected after their immune system had fully matured), may recognize virus protein HBZ as foreign, and HBZ-specific immune responses may be provoked. From this point of view, detection of HBZ-specific T cell responses might be expected in some HTLV-1 ACs, infected after becoming adults, but we did not see this in the present study.

In conclusion, we report the presence of HBZ-specific CD4 T cell responses in ATL patients who were in CR but only after allogeneic HCT. These responses potentially contribute to the