Human papillomavirus (HPV)-specific T-cell response to the HPV type 16 (HPV16) E6 protein has been shown to be associated with successful viral clearance. The patterns of CD8 T-cell epitopes within HPV16 E6 protein were previously studied in two women with HPV16 clearance. The goal of this study was to characterize these epitopes in terms of their minimal and optimal amino acid sequences and the human leukocyte antigen (HLA) restriction molecules. The presence of the epitope-specific memory T cells after viral clearance was also examined. In subject A, the dominant epitope was characterized to be E6 75–83 (KFSKYKISEY), restricted by the HLA-B62 molecule, while that of subject B was E6 133–142 (HNIRGRWTGR), restricted by the HLA-A6801 molecule. Homologous epitopes were identified in five other high-risk HPV types for both of these epitopes, but they were not recognized by respective T-cell clone cells. An enzyme-linked immunospot assay or tetramer analysis was performed on peripheral blood mononuclear cells from blood samples collected after viral clearance but prior to isolation of the T-cell clones. The presence of epitope-specific memory T cells was demonstrated. These data suggest that HPV-specific memory T cells were generated in vivo and that they may remain in circulation many months, if not years, after viral clearance. Our findings broaden the spectrum of the CD8 T-cell epitopes of the HPV16 E6 protein. The characterization of novel T-cell epitopes and long-lasting epitope-specific memory T cells may be useful for the development of a potential epitope-based vaccine.
ory T cells was examined using an ELISPOT assay or tetrramer staining, and the HPV-specific memory T cells appeared to remain in circulation many months, if not many years, after viral clearance.

**MATERIALS AND METHODS**

**Subjects and cell lines.** The generation of CD8 T-cell lines from the two women who were able to clear their HPV infections has been described previously (22). As a part of a longitudinal cohort study, they were monitored using cervical HPV DNA testing by PCR (27), cytology, and colposcopy every 4 months. Briefly, the CD8 T-cell lines were established by stimulating PBMCs using autologous dendritic cells (DCs) infected with recombinant vaccinia viruses expressing the HPV16 E6 protein. Using ELISPOT assays, the presence of potential CD8 T-cell epitopes in the E6 31–55 and E6 61–85 (dominant) regions was demonstrated for subject A (22) and that in the E6 31–55, E6 76–100, and E6 121–145 (dominant) regions was demonstrated for subject B (22). Each region was tested using a pool of three overlapping 15-mer peptides. The blood samples used to establish these CD8 T-cell lines were drawn 53 or 78 months after HPV16 clearance in subject A or B, respectively. The study protocol was approved by the University of California at San Francisco Committee on Human Research as well by the Institutional Review Board of the University of Arkansas for Medical Sciences.

**Synthetic HPV peptides.** A series of 15-mer peptides overlapping each other by 10 amino acids and a series of 9-mer peptides overlapping each other by 8 amino acids covering the HPV16 E6 protein have been described (20). To define the minimal and optimal amino acid sequences of the CD8 T-cell epitope, 8-mer, 9-mer, 10-mer, 11-mer, and homologous peptides (see Table 1) were synthesized as described previously (20, 21).

Isolation of antigen-specific T-cell clones after magnetic selection of IFN-γ-secreting cells. The CD8 T-cell lines from subjects A and B were stimulated for two additional 7-day cycles to increase the frequency of targeted antigen-specific T cells as described previously (20). Then, the HPV16 E6 antigen-specific CD8 T-cell lines (~2 × 10^5 cells from each line) were stimulated with 10 μM of each peptide contained in positive peptide pools (three 15-mer peptides contained in each peptide pool) for 3 to 6 h. Following this incubation, the IFN-γ-secreting cells were selected using a commercially available kit according to the manufacturer's instructions (IFN-γ secretion assay; Miltenyi Biotec). HPV16 E6 peptide-specific T-cell clones were isolated using a limiting dilution method as described previously (20, 21).

**ELISPOT assays for screening T-cell clones.** A previously described ELISPOT assay method (21) was used to screen possible epitope-specific T-cell clones. In short, the plate (MultiScreen; Millipore, Bedford, MA) was coated with 5 μg/ml anti-IFN-γ monoclonal antibody 1-D1K (Mabtech, Stockholm, Sweden) overnight at 4°C. After the plate was washed and blocked, 1 × 10^6 autologous Epstein-Barr virus-transformed B-lymphoblastoid cell line (LCL) cells were plated along with media containing T-cell clone cells (cell number not determined) to one of the two same position in triplicate (to test two peptide pools and a medium-only control for subject A) or quadruplicate (to test three peptide pools and a medium-only control for subject B) plates. The final concentration was 10 μM for each peptide. After 20 h of incubation, the wells were washed and incubated with biotin-conjugated anti-IFN-γ monoclonal antibody (1 μg/ml; Mabtech) for 2 h at 37°C. Then, the wells were washed and incubated with avidin-bound biotinylated horseradish peroxidase H (Vectastain Elite kit; Vector Laboratories, Inc., Burlingame, CA) for 1 h at 37°C. After additional washing of the wells, spots were developed using stable diaminobenzene (Research Genet-ics, Huntsville, AL) at room temperature. Spot-forming units were counted using an automated ELISPOT analyzer (Cell Technology, Inc., Jessup, MD). The wells that showed spots in an ELISPOT plate with one peptide pool, but not in other plates, were considered to potentially contain T-cell clones with the specificity of interest.

**ELISPOT assays to characterize the CD8 T-cell epitopes of the HPV16 E6 protein.** To confirm the specificity of the potential epitope-specific T-cell clones identified in screening, ELISPOT assays were repeated using 15-mer peptides contained in the positive peptide pools individually in duplicate or triplicate. One thousand T-cell clone cells were coincubated with 1 × 10^6 autologous LCL cells along with 20 U/ml of recombinant human interleukin-2 (rIL-2) in the presence of 15-mer peptide at a concentration of 10 μM. The ELISPOT assays were otherwise performed as described above. To determine the minimal and optimal amino acid sequences of the CD8 T-cell epitopes, additional ELISPOT assays were performed using peptides of various lengths. Serial dilutions of these peptides (10^–3 M to 10^–10 M) were also performed whenever necessary as described previously (21). A peptide of particular length was considered optimal if noticeably larger numbers of spot-forming units were observed at multiple concentrations.

To determine whether the HPV16 E6 epitopes were being endogenously processed, autologous LCL cells infected with recombinant vaccinia virus expressing the E6 protein (E6-vac) at a multiplicity of infection of 5 were used as antigen-presenting cells (APCs) in ELISPOT assays. The wild-type virus, Western Reserve (WR), and/or recombinant vaccinia virus expressing HPV16 E7 (E7-vac) served as negative controls. The ELISPOT assays were otherwise performed as described above.

**HLA typing.** HLA typing was performed at the University of California at San Francisco Immunogenetics Laboratory or at the University of Arkansas for Medical Sciences HLA Laboratory by a serological method or a PCR sequence-specific amplification method.

**Identification of the restricting HLA class I molecules.** To identify putative restricting HLA class I molecules, ELISPOT assays were performed, as described above, using allogeneic LCLs sharing one or a few class I molecules with the subjects. The results were confirmed using chromium release assays in which at least two LCLs expressing the putative HLA class I molecule were tested. The LCLs were labeled with 100 μCi of sodium chromate (Na_2_10^3CO_3) and were incubated with 10 μM of positive peptide (HPV16 E6 75–83 for subject A and E6 131–145 for subject B). After the cells were plated in a 96-well plate at 5 × 10^4/well, effector cells were added at multiple effector/target cell (E/T) ratios. After a 5-h incubation, the supernatants were harvested and radioactivity was counted with a gamma counter (Packard Instruments, Meriden, CT). The percent- age of specific lysis was calculated as previously described (23).

**Characterization of the surface phenotypes of the T-cell clones.** For characterization of the surface phenotypes of the T-cell clones, 5 × 10^5 T-cell clone cells were stained with CD4-phycocerythrin (PE)/CD8-fluorescein isothiocyanate (FITC) cocktail, CD3-FITC/CD16-PE cocktail, and corresponding isotype controls (Caltag, Burlingame, CA) for 30 min at 4°C. Then, the cells were washed and fixed. Events were acquired and analyzed using the Coulter EPICS XL-MCL flow cytometer (Beckman Coulter, Fullerton, CA).

**Examination of the recognition of homologous CD8 T-cell epitopes from other high-risk HPV types using ELISPOT assays.** Homologous epitopes, defined as peptides containing the same anchor residues (amino acid 2 and the last amino acid residue of the CD8 T-cell epitope) located within the 20-amino-acid region from the original HPV16 E6 epitopes, were identified by examining the protein sequences of the E6 proteins of HPV31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, and -73 (HPV Sequence Database, Los Alamos National Laboratory, University of California). Recognition of the homologous epitopes by the HPV16 E6 75–83- or E6 133-142-specific T-cell clones (1,000 cells per well) was tested using autologous LCL cells and LCL cells (1 × 10^5 LCL cells per well) from five additional individuals expressing the HLA-B*02 (for subject A) or HLA-A*68 (for subject B) molecule. The HPV16 E6 75-83 and E6 133-142 peptides were used as positive controls. Peptides were added at a concentration of 10 μM along with 20 U/ml of rIL-2, and the wells were set up in duplicate.

**Characterization of epitope-specific memory T cells by the ELISPOT assay and tetrramer analysis.** During the process of stimulating and isolating epitope-specific T-cell clones, the CD8 T cells from subjects A and B were incubated with autologous mature DCs infected with E6-vac since DCs are the most potent APCs. One drawback of this approach is that one cannot be certain whether the antigen-specific T cells are primed during the course of their natural HPV16 infection in vivo or during the course of the in vitro stimulation. If the former scenario is true, then the epitope-specific memory T cells may be detectable in PBMCs from the same subjects. In order to assess this possibility, a tetramer designed to bind T cells specific for the HPV16 E6 133–142 epitope restricted by the HLA-A*0601 molecule was obtained from the NIH tetramer facility for subject B. A tetramer could not be made for the HPV16 E6 75–83 epitope restricted by the HLA-B*02 molecule for subject A, since this HLA type was not available for production, and an ELISPOT assay was performed for the detection of epitope-specific T cells.

PBMCs from subject A (collected at 29 and 37 months) and from subject B (collected at 4, 22, 27, 48, and 74 months) after HPV16 clearance were available (Fig. 1). These PBMC samples were analyzed immediately after thawing (for subject B) and after in vitro stimulation in a manner known to enhance the number of memory T cells (10, 11, 15) (for subjects A and B). In short, cryopreserved PBMCs were thawed and incubated at 2 × 10^6/ml in medium (RPMI 1640 plus 10% human serum, 100 U/ml penicillin, and 100 μg/ml streptomycin) referred to as RP-10(18) overnight. Then, the cells were counted and were incubated at 1 × 10^5 PBMCs/ml in the presence of the respective HPV16 E6 peptide at a concentration of 10 μM (day 0). After a 3-day incubation, rIL-2 was added to each well to a concentration of 1,800 U/ml. One more milliliter of
FIG. 1. Natural history of HPV16 infection. (a) For subject A, the HPV infection was detected at one visit. The T-cell clones isolated to define the dominant CD8 T-cell epitope were derived from a blood sample drawn 53 months after HPV clearance. PBMCs collected at 29 and 37 months later (day 10), the cells were washed and cultured in RP-10H at 10^{6}/ml without peptide and rhIL-2 overnight. On the following day, 2.5 × 10^{5} PBMCs per well were used for an ELISPOT assay as described above for subject A. The percentage of epitope-specific T cells was calculated by subtracting the averaged spot-forming units of no-peptide control wells from those of peptide-containing wells and by dividing the difference by the total number of cells.

For subject B, various tetramer concentrations, incubation times, and incubation temperatures were examined. The concentration of 1:1,600 and incubation time of 30 min at room temperature were chosen. In addition, the PBMC samples were stained with CD4-FITC, CD14-FITC, CD19-FITC, CD8-PerCP (peridinin-chlorophyll-protein complex), and CD45RO-allophycocyanin (BD Biosciences, San Jose, CA). PBMCs from a healthy donor known to be HLA-A6801 negative were used as a negative control. T-cell clone cells (#83-B designates clone 83 from subject B) were mixed with PBMCs from this negative control donor (10% T-cell clone cells) and were used as the positive control.

On day 11, the samples were stained and analyzed using Becton Dickinson FACSCalibur (BD Bioscience). A lymphocyte gate was drawn in forward and side scatter, and this population was further gated for CD4/14/19-FITC negativity to eliminate nonspecific staining of CD4 T cells, monocytes, and B cells. One-hundred thousand events were acquired per sample. The percentages of tetramer-positive CD8+ T cells were calculated by dividing the number of cells in the circle in the right upper corner (region determined using the positive control sample) by the sum of cell numbers in the upper right and lower right quadrants (CD8 T cells). Whether these tetramer-positive CD8+ T cells were CD45RO+ T cells was also determined.

RESULTS

Natural history of HPV16 infection in subjects A and B. Subjects A and B were participants of a longitudinal study of HPV infection (18), and subject A’s HPV infection was detected at a single visit, and the blood used to establish the CD8 T-cell line and to isolate T-cell clones was drawn 53 months later. On the other hand, subject B had an HPV16 infection which lasted for over 4 years, and the blood sample was taken 78 months after clearance (Fig. 1).

HPV16 E6 71–85 region restricted by the HLA-B62 molecule; the dominant epitope characterized from subject A. Approximately 1.8 × 10^{5} (0.09%) IFN-γ-secreting cells were isolated from the CD8 T-cell line from subject A. Limiting dilution analysis was performed, and a total of 344 T-cell clones were expanded. A random selection of 94 T-cell clones were used for screening ELISPOT, and eight T-cell clones (# 8-A, #15-A, #40-A, #74-A, #76-A, #78-A, #93-A, and #94-A) were positive for the peptide pool covering the HPV16 E6 61–85 region (dominant region) (22), but none of the T-cell clones was positive for the peptide pool covering the E6 31–55 region (subdominant region) (22; data not shown). When the ELISPOT assay was repeated using individual 15-mer peptides, seven (#8-A, #15-A, #40-A, #74-A, #76-A, #78-A, and #94-A) of the eight screen-positive T-cell clones were positive with the E6 71–85 peptide but not with the E6 61–75 and E6 66–80 peptides (Fig. 2a). To examine the nature of the antigen processing, E6-vac- or WR-infected autologous LCL cells were used as APCs. The same seven T-cell clones were positive when tested with E6-vac-infected autologous LCL cells, suggesting that this E6 epitope is endogenously processed (Fig. 2b).

To determine the minimal and optimal epitope amino acid sequence, a series of ELISPOT assays were performed (Fig. 2c to e). Of the 9-mer peptides within the E6 71–85 region, the E6 75–83 9-mer peptide showed the most spot-forming units per well for all clones tested, followed by the E6 74–82 peptide (Fig. 2c). When the two 10-mer peptides surrounding the E6 75–83 peptide and two 8-mer peptides within were compared, the E6 75–83 9-mer peptide and the E6 74–83 10-mer peptide demonstrated comparable numbers of spot-forming units for all T-cell clones tested (Fig. 2d). The E6 74–83 9-mer peptide and the E6 75–84 10-mer peptide demonstrated considerably fewer spot-forming units. A serial dilution of the E6 75–83 9-mer peptide and the E6 74–83 10-mer peptide demonstrated comparable numbers of spot-forming units for all T-cell clones tested (Fig. 2d). The E6 74–83 9-mer peptide and the E6 75–84 10-mer peptide demonstrated considerably fewer spot-forming units. A serial dilution of the E6 75–83 9-mer peptide and the E6 74–83 10-mer peptide tested with clones #76-A and #78-A showed that they were equivalent over a wide range of peptide concentrations (Fig. 2e). Therefore, we designated E6 75–83 (9 amino acids) to be the minimal and optimal sequence for this epitope.

To identify the HLA restriction element of this novel epitope, a panel of allogeneic LCL cells matched to one or more HLA class I molecule(s) of subject A were used in an ELISPOT assay. An allogeneic LCL matched with the HLA-B62 molecule showed a positive response along with the autologous LCL cells for all four clones tested (#40-A, #76-A, #78-A, and #94-A) (data not shown). To confirm this result, a chromium release assay was performed with the T-cell clones (clones #76-A and #78-A) as effectors and LCL cells pulsed...
with the E6 75–83 peptide as targets. Three of the LCL cells were from allogeneic LCLs expressing the HLA-B62 molecule, and all of them were lysed by the T-cell clones (Fig. 2f), confirming that the restriction element of the HPV16 E6 75–83 epitope is the HLA-B62 molecule.

HPV16 E6 133–142 restricted by the HLA-A6801 molecule; the dominant epitope characterized from subject B. From this subject, $6.5 \times 10^6$ (0.31%) IFN-γ-secreting cells were selected, and 504 of 1,048 T-cell clones that grew were expanded. A random selection of 94 T-cell clones was screened using ELISPOT. Sixty-four of 94 T-cell clones tested were positive for the E6 121–146 region (dominant region) (22), and none of them was positive for the E6 31–55 and E6 76–100 regions (subdominant regions) (22; data not shown). Eight (#1-B, #4-B, 13-B8, #21-B, #74-B, #80-B, #83-B, and #87-B) of 64 screen-positive T-cell clones that grew well were retested with three individual 15-mer peptides in the region as well as with autologous LCL cells infected with E6-vac, E7-vac, or WR.

FIG. 2. Characterization of subject A’s dominant CD8 T-cell epitope in terms of its minimal and optimal amino acid sequence and the HLA-restricting molecule. The bars represent standard errors of the means. (a) ELISPOT assay performed to determine the specificity of the screen-positive T-cell clones to one of the three 15-mer peptides contained in the HPV16 E6 61–85 region. PHA, phytohemagglutinin. (b) ELISPOT assay performed to determine whether the epitope is endogenously presented using APCs infected with E6-vac or WR. (c) ELISPOT assay performed to identify the epitope within the 15-amino-acid region using 9-mer peptides overlapping by 8 amino acids. (d) ELISPOT assay performed to determine the optimal epitope sequence using two 10-mer peptides surrounding the E6 75-83 9-mer and two 8-mer peptides within it. The E6 74-82 9-mer peptide was also tested. (e) ELISPOT assay performed using serially diluted E6 75-83 9-mer peptide and E6 74-83 10-mer peptide ($10^{-5}$ M to $10^{-10}$ M) to determine the optimal peptide of minimum length. A representative (clone #78-A) of two T-cell clones tested is shown. (f) Chromium release assay performed to determine the restricting molecule for the HPV16 E6 75-83 epitope using peptide-pulsed autologous LCL cells or a panel of partially HLA-matched LCL cells as APCs. A representative (#78-A) of the two clones tested is shown. *: HLA type determined using one of the molecular methods.
eight T-cell clones were positive with the E6 131–145 peptide and E6-vac (Fig. 3a and b). As with the previous subject, a series of ELISPOT assays were performed to determine the optimal peptide of minimal length (Fig. 3c to e). Since none of the 9-mer peptides included in the E6 131–145 region was positive (data not shown), the ELISPOT assay was repeated with a series of 10-mer peptides, and only E6 133–142 was positive among the 10-mers (Fig. 3c). When two 11-mers surrounding this E6 133–142 10-mer and two 9-mers within it were tested, the 10-mer and the two 11-mers appeared equivalent (Fig. 3d). This was true even over a wide range of concentrations (Fig. 3e); therefore, the E6 133–142 10-mer peptide was designated to be the optimal peptide of minimal length.

A panel of allogeneic LCL cells matched to one or more HLA class I molecule(s) of subject B were used in an ELISPOT assay, and allogeneic LCL cells matched with the HLA-A68 molecule showed a positive response along with the autologous LCL cells for all eight clones tested (#1-B, #4-B, #13-B, #21-B, #74-B, #80-B, #83-B, and #87-B) (data not shown). To confirm this result, a chromium release assay was performed with the T-cell clones (clones #83-B and #87-B) as effectors and LCL cells pulsed with the E6 131–145 peptide as targets. Of the three HLA-A68-positive LCL cells, one was known to be HLA-A6801 while another one was known to be HLA-A6802 (Fig. 3f). The results were positive for autologous LCL cells and for LCL cells expressing A68 or A6801. Other allogeneic
TABLE 1. Amino acid sequences of high-risk HPV type homologous epitopes homologous for HPV16 E6 75–83 and E6 133–142 CD8 T-cell epitopes

| CD8 T-cell epitope homology and HPV type | Amino acid sequence | Position | Sequence length (no. of amino acids) |
|----------------------------------------|---------------------|----------|-------------------------------------|
| Homologous for HPV16                    |                     |          |                                     |
| E6 75–83 epitopes                       |                     |          |                                     |
| HPV16                                  | KFYSKISEY           | 75–83    | 9                                   |
| HPV33                                  | RFLSKISEY           | 68–76    | 9                                   |
| HPV51                                  | LFYSKIREY           | 68–76    | 9                                   |
| HPV52                                  | RFLSKISEY           | 68–76    | 9                                   |
| HPV56                                  | LFYSVKRKY           | 71–79    | 9                                   |
| HPV73                                  | KFYSKIREY           | 69–77    | 9                                   |
| Homologous for HPV16                    |                     |          |                                     |
| E6 133–142 epitopes                    |                     |          |                                     |
| HPV16                                  | HNIRGRWTRG          | 133–142  | 10                                  |
| HPV31                                  | HNIGGRWTRG          | 126–135  | 10                                  |
| HPV33                                  | HNISGRWAGR          | 126–135  | 10                                  |
| HPV51                                  | ANCWQRTRQR          | 137–146  | 10                                  |
| HPV52                                  | HNIMGRWTRG          | 126–135  | 10                                  |
| HPV58                                  | HNISGRWTRG          | 126–135  | 10                                  |

a Boldface amino acid residues are different from those in HPV16 E6 75–83 or E6 133–142 CD8 T-cell epitope.

LCL cells were negative, including the allogeneic LCL cells expressing the HLA-6802 molecule. Taken together, these data suggest that the restriction element of the HPV16 E6 133–142 epitope is the HLA-A6801 molecule.

No recognition of homologous epitopes from other high-risk HPV by the HPV16 E6 75-83-specific and E6 133-142-specific T-cell clone cells. To determine the potential recognition by epitope-specific T-cell clone cells, the presence of homologous epitopes from other high-risk HPV types were examined. Five homologous epitopes for the HPV16 E6 75–83 CD8 T-cell epitope (HPV33, -51, -52, -56, and -73) and five for the HPV16 133–142 epitope (HPV31, -33, -51, -52, and -58) were identified (Table 1). Their recognition by the HPV16 E6 75–83-specific T-cell clones (#76-A and #78-A) was examined by peptide-pulsed autologous LCL cells and five allogeneic LCL cells expressing the HLA-B62 molecule. All of the LCL cells were recognized, as measured by the number of spot-forming units in an ELISPOT assay when they were pulsed with the HPV16 E6 75–83 peptide but not with any other homologous epitopes (data not shown). The results were the same for the HPV16 E6 133-142 epitope in that the LCL cells were recognized only when pulsed with the HPV16 E6 133–142 peptide but not with the others (#83-B and #87-B) (data not shown). These data imply that the recognition of these HPV16 E6 epitopes is type specific.

HPV E6 75-83- and E6 133-142-specific T-cell clone cells show the surface phenotype of CD3+ CD4− CD8+ CD16−. The surface phenotypes of the E6 75-83- and E6 133-142-specific T-cell clones have been demonstrated to be CD3+ CD4− CD8+ CD16− (#40-A, #76-A, #78-A, #94-A, #1-B, #4-B, #13-B, #21-B, #74-B, #80-B, #83-B, and #87-B) (data not shown).

The presence of epitope-specific memory T cells in subjects A and B. Peptide-stimulated PBMC samples from subject A collected 29 and 37 months after HPV16 clearance (Fig. 1) demonstrated the frequencies of the epitope-specific T cells to be 0.039% and 0.092%, respectively (Fig. 4).

PBMC samples from subject B collected at 4, 22, 27, 48, and 74 months (Fig. 1) after the clearance of HPV16 infection were analyzed with and without in vitro stimulation with the HPV16 E6 133–142 peptide (Fig. 5 and Table 2). Three (one tetramer-positive CD8+ T-cell in 2,146 CD8 T cells at 4 months, one in 3,116 at 22 months, and one in 2,311 at 74 months) of the five PBMC samples (without in vitro stimulation) demonstrated staining for the tetramer-positive CD8+ T cells above that of the negative control (1 in 5,010). Upon stimulation with the HPV16 E6 133–142 peptide, the frequencies of these tetramer-positive CD8+ T cells increased noticeably in the PBMC samples taken at 4 months and 22 months (1 in 512 and 1 in 510, respectively). Ninety-eight percent and 100% of these tetramer-positive CD8+ T cells were CD45RO+ in these samples, respectively (data not shown). Taken together, the HPV16 E6 133-142-specific memory T-cells were detectable at 4 months and 22 months after HPV16 clearance.

DISCUSSION

In the present study, we characterized HPV16-specific CD8 T-cell epitopes from two women in whom the presence of potential CD8 T-cell epitopes was demonstrated after HPV16 clearance (22). For one subject (subject A), the duration of the HPV16 infection was brief and it was detectable only at a single visit, while that of the other subject (subject B) lasted for over 4 years (Fig. 1). Although both subjects demonstrated the presence of potential HPV16 E6 CD8 T-cell epitopes within more than one region, only the dominant T-cell epitopes were characterized since we were unsuccessful in isolating T-cell clones from the subdominant regions. These dominant epitopes were characterized to be the HPV16 E6 75–83 epitope restricted by the HLA-B62 molecule and the HPV16 E6 133–142 epitope restricted by the HLA-A6801 molecule. The antigen frequencies of the HLA-B62 molecule are 0.9%, 6.2%, and 18.1% in African, Caucasian, and Asian populations, respectively, and those of the HLA-A68 molecule are 19.4%, 8.0%, and 2.6% in the respective populations (17). Previously, we had characterized HPV16-specific CD8 T-cell epitopes from the same cohort of women (20, 22) and described the phenom-
The utility of the newly described HPV16 E6 CD8 T-cell epitopes as the source of antigens for peptide-based vaccine or immunotherapy would be broader if the epitope-specific T cells also recognized homologous epitopes from other high-risk HPV types. To evaluate whether these two newly described CD8 T-cell epitopes may be similar enough to potential epitopes from other high-risk HPV types, recognition of homologous epitopes from HPV35, -39, -45, -51, and -73 by HPV16 E6 52-61-specific T-cell clone cells was shown (16). The utility of the newly described HPV16 E6 CD8 T-cell epitopes as the source of antigens for peptide-based vaccine or immunotherapy would be broader if the epitope-specific T cells also recognized homologous epitopes from other high-risk HPV types. To evaluate whether these two newly described CD8 T-cell epitopes may be similar enough to potential epitopes from other high-risk HPV types, recognition of homologous epitopes from HPV35, -39, -45, -51, and -73 by HPV16 E6 52-61-specific T-cell clone cells was examined. For these epitopes, no recognition was demonstrated, suggesting that these two T-cell epitopes are type specific. These results are in contrast to the HPV16 E6 52–61 epitope, which was evaluated by the same method, in which the recognition of homologous epitopes from HPV35, -39, -45, -51, and -73 by HPV16 E6 52-61-specific T-cell clone cells was shown (16).

Memory T cells play an important role in maintaining long-term immunity to previously encountered pathogens or tumor antigens. They may proliferate, rapidly acquire effector functions to kill virus-infected cells or tumor cells, or secrete cytokines that inhibit replication of the pathogen after restimulation with reexposure to antigen (14). For subject A, the epitope-specific memory T cells were detected in circulation 37 months after viral clearance using an ELISPOT assay (Fig. 4). For subject B, tetramer-positive CD8^+ T cells were present ex vivo in three of the five PBMC samples (4, 22, and 74 months). Upon peptide stimulation in vitro for 10 days, the frequencies of tetramer-positive CD8^+ T cells expanded in two of the three PBMC samples (4 and 22 months; Table 2), and almost all of the epitope-specific T cells were CD45RO^+. There is no good
explanation as to why PBMCs collected at 74 months were not confirmed to be positive after peptide stimulation, while those collected at 4 and 22 months were. One possibility is that there is no a sufficient difference in the frequencies of the tetramer-positive PBMCs (0.043% at 74 months) and the negative control (0.020%), such that the samples that appear positive (4, 22, and 74 months) are actually in a gray zone between positive and negative. This is a reflection of a low number of circulating HPV-specific CD8 T cells, especially long after viral clearance. Since this method of in vitro stimulation is used to increase the number of memory T cells (10, 11, 15), the data seem to indicate that the HPV-specific memory T cells can certainly be detected up to 37 months, or 3 years, after the HPV DNA has become undetectable. Furthermore, the fact that epitope-specific memory cells were detected prior to isolation of T-cell clones suggests that they were generated in vivo at the time of HPV16 infection rather than in vitro. It may be that the HPV16 E6 132-142-specific T cells were isolated 78 months after HPV16 clearance because mature DCs, which are known to be the most potent APCs, were able to amplify the number of epitope-specific memory T cells to a level at which they can be isolated. Godkin et al. demonstrated a memory T-cell response to hepatitis virus epitopes many years after viral clearance (10), and Demkowicz et al. showed persistence of T cells specific for vaccinia virus for up to 50 years in the absence of reexposure (5). The challenge in the future is to determine whether these memory cells confer long-term protection to HPV reinfection and whether these memory T cells are capable of quickly differentiating into a potent effector response during HPV reinfection.

Tetramers specific for HPV16 E6 or E7 CD8 T-cell epitopes have been used by other investigators to examine the frequencies of HPV-specific T lymphocytes in patients with cervical cancer (30, 31), with cervical intraepithelial neoplasia grade 3 (CIN 3) (30), and with squamous cell carcinoma of the oropharynx (SCCO) (13). In T-cell lines established from five HLA-A*0201-positive patients with HPV16-positive cervical cancer by stimulation with the HPV16 E7 11–20 peptide, 1 to 12% of cells were tetramer positive (30). However, ex vivo analysis of PBMCs from patients with CIN 3 (1 of 1,260 to 1 of 19,073) and normal controls (1 of 1,855 to 1 of 42,004) revealed low frequencies of tetramer-positive CD8⁺ cells in both. Similar findings were reported by Hoffmann and colleagues in that the frequencies of tetramer-positive CD8⁺ cells for three epitopes (HPV16 E7 11–20, E7 82–90, and E7 86–93) studies were not significantly different between patients with SCCO and healthy controls (13). However, a significant difference was found in frequencies of the HPV16 E7 11-20-specific T cells between patients with SCCO whose tumors were positive for the expression of HPV16 E7 and p16 and who were negative for these two proteins. Zehbe et al. examined the presence of HPV16 E6 11-29-specific T cells in PBMCs, tumor-infiltrating lymphocytes, and in lymph nodes of five HLA-A*0201-positive patients with HPV16-positive cervical cancer (31). Interestingly, the percentage of tetramer-positive CD8⁺ cells was highest in the tumor-infiltrating lymphocytes but they had a naive phenotype (CD45RA⁺CCR7⁺). To our knowledge, our group is the first to examine the presence of HPV-specific T lymphocytes using a tetramer in a woman whose HPV16 infection and its clearance were documented.

Vaccination with HPV16 E7 CTL epitopes was shown to elicit efficient antitumor CTL responses and to prevent the outgrowth of a lethal dose of HPV16-induced tumor cells in mice, indicating the feasibility of peptide immunization (7, 25). However, the use of peptides as HPV therapeutic vaccines in human clinical trials has shown limited success. Ressing et al. (26) used two CTL epitopes, HPV16 E7 11–20 and E7 86–93, and a universal T-helper epitope to perform a peptide-based phase I/II vaccination trial to induce antitumor immune responses in patients with recurrent or residual cervical carcinoma. Though no HPV16-specific CTL response was detectable, strong T-helper peptide-specific proliferations were detected in 4 of 12 patients, showing T-helper epitopes may also play a role in the generation of protective antitumor immunity. In another trial, 18 women with high-grade cervical or vulvar intraepithelial neoplasia II/III who were positive for HPV16 received vaccination with synthetic E7 12–20 peptide representing the CTL epitope (19). The E7 86–93 peptide linked to a helper T-cell epitope peptide was administered to part of the women at the same time. It was demonstrated that six patients had partial colposcopically measured regression of their CIN lesions, and three patients completely cleared their dysplasia after vaccine. Similarly, an epitope-based HPV therapeutic vaccine delivered in a form of a DNA vaccine has shown partial success. ZYC101a contains a plasmid encoding portions derived from the HPV16 and -18 E6 and E7 proteins. In a phase II clinical trial that enrolled 127 evaluable subjects with CIN 2/3 (9), a higher percentage of subjects receiving ZYC101a demonstrated regression compared to placebo (45% versus 27%). However, the difference was not statistically significant (P = 0.12). In a subgroup of women ≤25 years old (n = 43), the regression rate was significantly higher in the vaccine group than in the placebo group (70% versus 23%; P = 0.007). In short, we described two novel HPV16 E6 CD8 T-cell epitopes from women who were able to clear their HPV16 infection and demonstrated the presence of HPV-specific memory T cells years after its clearance. The challenges for the future, in order to translate these findings to the development of effective vaccine, are to evaluate more efficient methods of presenting the T-cell epitopes so the clinical response rates would be more robust.

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