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T-Cell Response to Human Papillomavirus Type 58 L1, E6, and E7 Peptides in Women with Cleared Infection, Cervical Intraepithelial Neoplasia, or Invasive Cancer[▽]

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Human papillomavirus type 58 (HPV-58) exists in a relatively high prevalence in certain parts of the world, including East Asia. This study examined the T-cell response to HPV-58 L1, E6, and E7 peptides among women with cleared infection, cervical intraepithelial neoplasia grade 2 (CIN2) or CIN3, or invasive cervical cancer (ICC). Peptides found to be reactive in the *in vitro* peptide binding assay or mouse-stimulating study were tested with a gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay to detect peptide-specific responses from the peripheral blood mononuclear cells (PBMC) collected from 91 HPV-58-infected women (32 with cleared infection, 16 CIN2, 15 CIN3, and 28 ICC). Four HLA-A11-restricted HPV-58 L1 peptides, located at amino acid positions 296 to 304, 327 to 335, 101 to 109, and 469 to 477, showed positive IFN-γ ELISPOT results and were mainly from women with cleared infection. Two HLA-A11-restricted E6 peptides (amino acid positions 64 to 72 and 94 to 102) and three HLA-A11-restricted E7 peptides (amino acid positions 78 to 86, 74 to 82, and 88 to 96) showed a positive response. A response to E6 and E7 peptides was mainly observed from subjects with CIN2 or above. One HLA-A2-restricted E6 peptide, located at amino acid position 99 to 107, elicited a positive response in two CIN2 subjects. One HLA-A24-restricted L1 peptide, located at amino acid position 468 to 476, also elicited a positive response in two CIN2 subjects. In summary, this study has identified a few immunogenic epitopes for HPV-58 E6 and E7 proteins. It is worthwhile to further investigate whether responses to these epitopes have a role in clearing an established cervical lesion.

Infection with high-risk human papillomaviruses (HPV) is a necessary cause for cervical cancer (14, 21, 33). Of the more than 40 HPV types that can infect the female genital tract, at least 15 have been shown to be associated with an increased risk for cancer development (21), and HPV type 16 (HPV-16) and HPV-18 account for more than 70% of cervical cancers detected worldwide (6, 10, 28). Currently, two vaccines have been approved for clinical use to prevent HPV infection and its potential consequences of developing cervical intraepithelial neoplasia (CIN) and invasive cervical cancer (ICC) (27). Both vaccines are prophylactic and contain HPV-16 and HPV-18 L1 protein-based virus-like particles. To date, the development of therapeutic vaccines has been restricted to early-phase clinical trials, and none has been approved for clinical use. HPV-specific cytotoxic cellular immunity against the E6 and E7 proteins that are consistently expressed in cervical neoplasia is important in clearing the established lesions. The results of initial studies using *in vitro* and *in vivo* models demonstrated that viral peptides were able to stimulate murine cytotoxic T-lymphocytes, and these peptides were also able to stimulate human cytotoxic T-lymphocytes against HPV-positive cervical cell lines (1, 13, 25). These findings have led to the search for peptides that can elicit or augment an HPV-specific cytotoxic cellular response from patients who have developed preinvasive or invasive cervical neoplasia (4, 29, 35). While HPV-16 and HPV-18 are the most prevalent HPV types found in cervical cancers detected worldwide, HPV-58 has been found at a relatively higher frequency in East Asia (5, 7). Currently, data on T-cell response to HPV-58 peptides are not available; therefore, the aim of this study was to characterize the T-cell response against peptides derived from the L1, E6, and E7 proteins of HPV-58 among women with cleared infection or cervical neoplasia.

MATERIALS AND METHODS

HLA-11 peptide selection by *in vitro* peptide binding assay. The HLA-A*1101 (HLA-A11) heavy (H) chain and B2M were refolded in the presence of the synthetic peptides (Table 1). The resultant complexes were then analyzed by a sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, the HLA-A11 H chain and B2M were mixed with each candidate peptide at a molecular ratio of 1:1:3. After incubation at 4°C for 24 to 48 h, the soluble portion was concentrated using a Centricon centrifugal filter unit (YM100; Millipore, Billerica, MA), followed by ELISA analysis.

An *in vitro* ELISA-based peptide-binding assay was conducted based on a protocol reported previously, with minor modifications (9). Briefly, a 96-well microtiter plate coated with 5 μg/ml of anti-HLA antibody (W6/32) in 100 mM carbonate buffer (pH 9.6) was incubated at 4°C overnight. After blocking with 10% nonfat milk in phosphate-buffered saline (PBS), the concentrated refolded

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TABLE 1. Results of *in vitro* peptide binding assay for HLA-A11-restricted cytotoxic-T-lymphocyte epitopes derived from HPV-58 E6, E7, and L1 proteins

Protein	Peptide sequence	Amino acid position	Relative binding ability (%)
HPV-58 L1	SIKSPNNNK	77–85	249.9
	RVRLPDPNK	101–109	387.3
	AVPDDLYIK	296–304	406.2
	TSESQLFNK	327–335	126.9
	MTLCTEVTK	368–376	332.3
	EVTKEGTYK	373–381	23.2
	GTYKNDNFK	378–386	321.6
	LQFVFQLCK	397–405	255.7
	YTFWEVNLK	469–477	181.9
	LLQSGLKAK	494–502	7.0
	PTTRAPSTK	510–518	48.6
	RAPSTKRKK	513–521	9.8
	STKRKKVKK	516–524	145.6
HPV-58 E6	QALETSVHE	17–25	0
	TSVHEIELK	21–29	58.7
	KVCLRLLSK	64–72	142.2
	SLYGDTLEQ	82–90	0
	DTLEQTLKK	86–94	7.6
	KCLNEILIR	94–102	56.4
	RPLCPQEKK	108–116	3.2
HPV-58 E7	CYTCGTTVR	59–67	10.4
	LCINSTATE	68–76	0
	ATEVRTLQQ	74–82	0
	CTIVCPSCA	88–96	0.8
	TIVCPSCAQ	89–97	3.3
EBV EBNA3B (positive control)	IVTDFSVIK	416–424	100

TABLE 2. Results of mouse immunization study for HLA-A24-restricted cytotoxic-T-lymphocyte epitopes derived from HPV-58 L1 and E6 proteins

Protein	Peptide sequence	Amino acid position	No. of IFN-γ spots/1 × 10^6 cells[a]
HPV-58 E6	VYDFVFADL	42–50	155
	DFVFADLRI	44–52	0
	VFADLRIVY	46–54	48
	VYRDGNPFA	53–61	0
	PFAVCKVCL	59–67	0
	VCLRLLSKI	65–73	0
	EYRHYNYSL	75–83	0
HPV-58 L1	EYVSRTSIY	52–60	0
	YYYAGSSRL	60–68	0
	YYAGSSRLL	61–69	0
	QYRVFRVRL	96–104	0
	VRLPDPNKF	102–110	0
	FYNPDTQRL	117–125	0
	DYKQTQLCL	117–185	0
	FFPTPSGSI	317–325	348
	EYVRHVEEY	387–395	0
	VFQLCKITL	400–408	15
	TYIHTMDSN	414–422	7
	TYRFVTSQA	442–450	39
	KYTFWEVNL	468–476	103
	KFSADLDQF	479–487	0
EBV LMP-2	TYGPVFMCL	419–427	101

[a] The data represent the average numbers of spots from three mice.

complexes were added, and horseradish peroxidase (HRP)-conjugated rabbit anti-human B2M (1:2,500) was used to detect complex formation. The enhancing reagent EnVision-HRP (Dako, Denmark) was used to amplify the signal. The substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was used for color development, which was stopped with 1 N sulfuric acid. Optical density was measured at 450 nm using a plate reader (Molecular Devices, Sunnyvale, CA).

HLA-A2 and -A24 peptide selection by immunization of HLA-A2 or HLA-A24 transgenic mice. One-milligram amounts of the indicated nonamers (Tables 2 and 3) and 1 mg of PADRE peptide (AKFVAAWTLKAAA) in 0.5 ml PBS were mixed well with 0.5 ml incomplete Freund's adjuvant (IFA). The positive-control peptides for HLA-A2 and HLA-A24 were from HPV-16 E7 and Epstein-Barr virus (EBV) LMP-2, respectively. One hundred microliters of each mixture was subcutaneously injected into the tail base of HLA-A2- or HLA-24-transgenic mice twice at a 7-day interval. Spleen cells were harvested 7 days after the second injection and cultured with 10 g/ml of peptides for 48 h, followed by a gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay (eBioscience, San Diego, CA). The wells of 96-well plates with nitrocellulose membrane inserts were coated with 50 μl of anti-IFN-γ antibody solution (clone AN18, 10 μg/ml in 1× PBS; eBioscience) and incubated for 18 h at 4°C. The plates were then washed with PBS four times and blocked with 100 μl of RPMI medium supplemented with 10% fetal bovine serum (FBS) (RPMI-10) per well for 1 to 3 h to prevent nonspecific binding in later steps. Next, 2×10^5 or 5×10^5 splenocytes with 10 μg/ml of the indicated peptides were added to the plate at a final volume of 200 μl of RPMI-10 (Tables 2 and 3). The ELISPOT assay was performed in triplicate for each experimental condition. Specifically, the plates were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 48 h. After this incubation, the cells were removed from the plate by washing six times with 0.05% (wt/vol) Tween 20 in PBS. A 50-μl aliquot of biotinylated anti-IFN-γ antibody (clone R46A2, 2 μg/ml in PBS; eBioscience) was then added to each well. The plates were again incubated in a humidified atmosphere of 5% carbon dioxide in air at 37°C for 2 h. The washing steps were repeated. After an hour of incubation with an avidin-HRP complex reagent (eBioscience) at room temperature, the plates were washed again three times with 0.05% (wt/vol) Tween 20 in PBS and then three times with PBS alone. A 100-μl aliquot of 3-amine-9-ethyl carbazole (ACE; Sigma-Aldrich, St. Louis, MO) staining solution was added to each well to develop the spots. The reaction was stopped after 4 to 6 min by placing the plate under tap water. The spots were then counted using an ELISPOT reader (Cellular Technology Ltd., Shaker Heights, OH).

Study subjects. Women with abnormal cytology results were recruited from the colposcopy clinic of Prince of Wales Hospital in Hong Kong from 2006 to

TABLE 3. Results of mouse immunization study for HLA-A2-restricted cytotoxic-T-lymphocyte epitopes derived from HPV-58 E6 and E7 proteins

Protein	Peptide sequence	Amino acid position	No. of IFN-γ spots/1 × 10^6 cells[a]
HPV-58 E6	TLHDLCQAL	11–19	0
	ALETSVHEI	18–26	64
	FVFADLRIV	45–53	0
	AVCKVCLRL	61–69	0
	VCLRLLSKI	65–73	37
	RLLSKISEY	68–76	0
	TLEQTLKKC	87–95	0
	TLKKCLNEI	91–99	0
	ILIRCIICQ	99–107	17
HPV-58 E7	TLREYILDL	7–15	0
	DLHPEPTDL	14–22	0
	CINSTATEV	69–77	13
	STATEVRTL	72–80	0
	EVRTLQQLL	76–84	0
	TLQQLLMGT	79–87	0
	QLLMGTCTI	82–90	0
HPV-16 E7	YMLDLQPETT	11–20	25

[a] The data represent the average numbers of spots from three mice.

TABLE 4. HPV-58 peptides used in IFN-γ ELISPOT study for subjects with cleared infection or cervical neoplasia

Peptide sequence	HLA restriction type	Gene region, amino acid position in HPV-58[a]	% Similarity[b] to corresponding peptide in HPV-16/-18
SIKSPNNNK	A11	L1, 77–85	HPV-16, L1 77–85, 78
RVRLPDPNK	A11	L1, 101–109	—
AVPDDLYIK	A11	L1, 296–304	HPV-16, L1 296–304, 89
TSESQLFNK	A11	L1, 327–335	HPV-18, L1 362–370, 89
MTLCTEVTK	A11	L1, 368–376	—
GTYKNDNFK	A11	L1, 378–386	—
LQFVFQLCK	A11	L1, 397–405	—
YTFWEVNLK	A11	L1, 469–477	HPV-16, L1 470–478, 100
STKRKKVKK	A11	L1, 516–524	—
TSVHEIELK	A11	E6, 21–29	—
KVCLRLLSK	A11	E6, 64–72	—
KCLNEILIR	A11	E6, 94–102	—
CYTCGTTVR	A11	E7, 59–67	—
ATEVRTLQQ	A11	E7, 74–82	—
LCINSTATE	A11	E7, 68–76	—
CTIVCPSCA	A11	E7, 88–96	—
TIVCPSCAQ	A11	E7, 89–97	—
VYDFVFADL	A24	E6, 42–50	—
VFADLRIVY	A24	E6, 46–54	—
FFPTPSGSI	A24	L1, 317–325	HPV-16, L1 317–325, 78
VFQLCKITL	A24	L1, 400–408	—
TYIHTMDSN	A24	L1, 414–422	HPV-16, L1 415–423, 67
TYRFVTSQA	A24	L1, 442–450	HPV-16, L1 443–451, 100
KYTFWEVNL	A24	L1, 468–476	HPV-16, L1 469–477, 100
ALETSVHEI	A2	E6, 18–26	—
VCLRLLSKI	A2	E6, 65–73	—
ILIRCIICQ	A2	E6, 99–107	—
CINSTATEV	A2	E7, 69–77	—

[a] Amino acid position numbering is according to the HPV-58 prototype (GenBank accession no. D90400).
[b] —, similarity was <60%.

TABLE 5. Positive peptide pool used in IFN-γ ELISPOT study for subjects with cleared infection or cervical neoplasia

HLA restriction type	Peptide sequence	Amino acid position	Virus,[a] protein
A2	VLGPISGHV	14–22	CMV, pp65
A2	MLNIPSINV	120–128	CMV, pp65
A2	NLVPMVATV	495–503	CMV, pp65
A2	RIFAELEGV	522–530	CMV, pp65
A11	AVFDRKSDAK	399–408	EBV, EBNA3B
A11	GPISGHVLK	16–24	CMV, pp65
A11	ATVQGQNLK	501–509	CMV, pp65
A24	RYSIFFDY	246–253	EBV, EBNA3A
A24	TYGPVFMCL	419–427	EBV, LMP2
A24	VYALPLKML	113–121	CMV, pp65

[a] EBV, Epstein-Barr virus; CMV, cytomegalovirus.

2008. In addition, patients with ICC were also recruited from the oncology clinic of the same hospital. The remainders of cervical scrape samples submitted for routine cytological examination were used for HPV detection and typing. Briefly, HPV DNA was detected by a single-round PCR based on the PGMY09/11 primer set according to a method described previously (8). To identify HPV-58-positive subjects, samples positive for HPV DNA were either typed by restriction fragment length polymorphisms or by using a linear array HPV genotyping kit (Roche Molecular Diagnostics, CA), as described previously (5, 8).

The cervical disease status was confirmed by histology. Patients who had no abnormalities detected by colposcopy and, hence, did not have biopsy specimens taken were followed up by cytology every 6 months. A cleared, self-limiting HPV-58 infection was defined as having had HPV-58 detected in the first visit and becoming negative for at least two subsequent follow-up visits, with the cytology results returned to normal.

Subjects who were found to be positive for HPV-58 and with a cervical disease status confirmed by histology or with a cleared infection were invited to donate blood for HLA typing and T-cell epitope mapping. The HLA class 1A alleles of the study subjects were identified using the AccuPlex typing kit (Dynal Biotech, Wirral, United Kingdom). This kit provides low-resolution typing. Briefly, peripheral blood mononuclear cells (PBMC) were used as a source of genomic DNA for PCR amplification using locus-specific primers. The biotinylated PCR products were hybridized to sequence-specific oligonucleotide probes, with the final signals recorded by a Luminex flow analyzer. AccuMatch software was used for HLA assignment. Subjects who belonged to the three most common HLA types (A11, A24, or A2) were selected for T-cell epitope study.

All study subjects provided informed consent. The study was approved by the local institutional ethics committee.

Isolation of PBMC. PBMC were isolated by Ficoll-Paque Plus gradient centrifugation (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Recovered PBMC were washed, assessed for viability by trypan blue exclusion, and cryopreserved at 1×10^7 cells/ml in freezing medium containing 10% dimethyl sulfoxide (Sigma, St. Louis, MO) and 90% FBS (HyClone, MA).

Peptide stimulation of PBMC. Peptides showing positive results from the *in vitro* binding assay or mouse immunization study were selected for IFN-γ ELISPOT assay (Table 4).

Cryopreserved PBMC were thawed and washed twice with AIM-V-RPMI 1640 containing 2 mM glutamine, 25 mM HEPES (Invitrogen, Carlsbad, CA) supplemented with 50 μM 2-mercaptoethanol (Sigma, St. Louis, MO), 0.1 μM nonessential amino acid solution (Invitrogen, Carlsbad, CA), and 10% human AB serum (Sigma, St. Louis, MO). The viable cells were counted using trypan blue exclusion and resuspended at 3×10^5 cells/ml. One milliliter of the PBMC suspension was added to a 24-well plate (Costar; Corning Incorporated, NY) before the addition of the HLA-matched peptides, each at a concentration of 10 μg/ml. For the positive control, PBMC were cultured with an HLA-A2-, -A11-, or -A24-restricted positive peptide pool containing peptides from EBV and human cytomegalovirus (CMV) at 10 μg/ml each (Table 5). The PBMC were prestimulated for a total of 4 days at 37°C. On day 3, 1 ml of AIM-V-RPMI 1640 medium containing 20 IU/ml recombinant interleukin-2 (AbD Serotec, Oxford, United Kingdom) was added. On the next day (day 4), 1 ml of culture medium was removed and replaced with 1 ml of fresh AIM-V-RPMI 1640, and the culture was further incubated at 37°C for 18 to 20 h.

IFN-γ ELISPOT assay. On day 5, peptide-pulsed PBMC were harvested and washed once, and the viable cells counted using trypan blue exclusion. The cell concentration was adjusted to give 2×10^5 cells/ml. The wells of a multiscreen 96-well plate (Millipore, Billerica, MA) that was prewetted with 35% alcohol were washed three times with PBS (Invitrogen, Carlsbad, CA), coated with 100 μl of IFN-γ capture antibody (R&D Systems, Minneapolis, MN), and left overnight at 4°C. On the next day, before use, the plate wells were washed four times with PBS and blocked at room temperature with 200 μl of AIM-V-RPMI 1640 medium containing 10% FBS for at least 2 h before PBMC seeding. PBMC that had been prestimulated with the appropriate peptides were seeded in triplicates at 10^4 cells per well. A total of 10^4 autologous PBMC that acted as antigen-presenting cells (APCs) were also added to each test well. The total volume of medium per well was 100 μl. Fresh peptides, each at a concentration of 10 μg/ml, were added to each well. For the positive controls, PBMC that had been pulsed with the HLA-A2-, -A11-, or -A24-restricted positive peptide pool were cultured again with a fresh positive peptide pool at 10 μg/ml each (Table 5). PBMC stimulated with concanavalin A (Calbiochem, United States) at a concentration of 0.5 μg/ml were used as a positive control for lymphocyte activation. Negative-control wells contained only PBMC that had gone through 4 days of incubation in the absence of peptide. For background controls, only APCs were incubated. The 96-well ELISPOT plate was wrapped in aluminum foil to maintain well-to-well reproducibility and to minimize background staining and was incubated at 37°C for a further 18 to 20 h. The ELISPOT plate was washed four times with 0.05% Tween 20–PBS, and then 100 μl of human IFN-γ detection antibody was added (R&D Systems, Inc., Minneapolis, MN) and incubated at 4°C for 12 to 16 h. After this, the washings were repeated and 100 μl of streptavidin-alkaline phosphatase (R&D Systems, Inc., Minneapolis, MN) in 10% FBS diluted with

PBS was added to each well. This was further incubated at room temperature for 2 h. The plate was washed again with 0.05% Tween 20–PBS, and 100 μl of 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (BCIP-NBT) chromogen (R&D Systems, Inc., Minneapolis, MN) was added and incubated for 30 min for color development. The reaction was stopped by washing six times with distilled water. The spots were counted using a cytotoxic-T-lymphocyte ImmunoSpot S4 UV analyzer (C.T.L. Cellular Technology Ltd., United States).

Criteria used in previously published studies were adopted to assign a specific response (29, 32). The net spot count for each peptide was obtained by subtracting the average results for background controls that contain only APCs from the total number of spots observed in the test well. For a significant T-cell response, the mean net spot count in the peptide test wells must have been greater than the mean plus twice the standard deviation of those of the negative-control wells and at the same time greater than 10 spots.

RESULTS

Peptide selection. Altogether, nine HLA-A11-restricted L1 peptides and three A11-restricted E6 peptides showed a relative binding ability greater than 50% of that of the positive control (Table 1). In the mouse immunization study, five A24-restricted L1 peptides, two A24-restricted E6 peptides, three A2-restricted E6 peptides, and one A2-restricted E7 peptide showed positive results (Tables 2 and 3). These peptides were selected for further IFN-γ ELISPOT study using human PBMC samples. In addition, five A11-restricted E7 peptides with high scores from bioinformatic prediction were also included (Table 4). Of these peptides selected for further study, three L1 peptides showed 100% similarity with HPV-16 L1 (Table 4).

Subject distribution. A total of 91 subjects were recruited for the T-cell response study using the IFN-γ ELISPOT assay. Thirty-two patients aged 36 to 56 (mean, 45.3; standard deviation [SD], 10.3) years had cleared HPV-58 infection, with cytology returning to normal, and had become HPV negative on follow-up. Thirty-one patients aged 32 to 75 (mean, 43.6; SD, 11.6) years had histology-confirmed CIN (16 CIN2 and 15 CIN3). Twenty-eight patients aged 31 to 77 (mean, 59.5; SD, 12.4) years had ICC (27 squamous cell carcinoma and 1 undifferentiated carcinoma).

HPV-58 infection. All 91 study subjects had HPV-58 DNA detected from their cytology specimens. Altogether, 73 subjects had HPV-58 as a single infection (25 cleared infections, 12 CIN2, 12 CIN3, and 24 ICC), and 18 subjects had HPV-58 detected together with one or more other HPV types (7 cleared infections, 4 CIN2, 3 CIN3, and 4 ICC). The proportions of single versus coinfections among women with different cervical states were not significantly different (*P* value of 0.826 by Chi-squared test).

HLA-A11-specific T-cell epitopes identified from human PBMC. Altogether, 53 subjects (17 cleared infections, 10 CIN2, 10 CIN3, and 16 ICC) either homozygous or heterozygous for HLA-A11 were studied. The results showed four L1 peptides with a positive response in the IFN-γ ELISPOT assay. These four peptides were located at amino acid positions 101 to 109, 296 to 304, 327 to 335, and 469 to 477 of the HPV-58 L1 protein (L1 101–109, 296–304, 327–335, and 469–477 peptides) (Table 4). The mean net number of IFN-γ spots detected ranged from 12 to 54 per 10^4 cells. All peptides, except L1 296–304, only had positive signals detected from the PBMC collected from patients with cleared infection. The positive rate among subjects with cleared infection was 41.2% for peptide L1 101–109, 11.8% for L1 296–304, 35.3% for L1 327–335, and 47.1% for L1 469–477.

Two E6 peptides, located at amino acid positions 64 to 72 and 94 to 102, were found to elicit positive responses for HLA-A11 subjects. One (5.9%) subject with cleared infection, two (20.0%) CIN2 subjects, and four (25.0%) ICC subjects had positive responses to the E6 64–72 peptide. Although a higher response rate was found among CIN2 and ICC subjects, the difference was not statistically significant. The E6 94–102 peptide showed positive responses only in two of the 10 CIN2 subjects and in none of the subjects with cleared infection, CIN3, or ICC (Table 6).

Three peptides located at amino acid positions 74 to 82, 78 to 86, and 88 to 96 of the HPV-58 E7 protein elicited positive responses in the IFN-γ ELISPOT assay. The E7 78-86 peptide showed positive responses from two (20.0%) CIN2 subjects, and the E7 88-96 peptide showed positive responses from two (12.5%) ICC subjects, while the E7 74-82 peptide showed positive responses across all grades of cervical lesions, including three (17.6%) cleared infections, four (40.0%) CIN2, two (20.0%) CIN3, and three (18.8%) ICC (Table 6).

Of the 10 HLA-A11 subjects with coinfections, four had positive responses (two subjects with cleared infection responded to L1 101-109, 327-335, and 469-477; another subject with cleared infection responded to L1 327-335; and a subject with CIN2 responded to L1 296-304). The positive response rates among groups with single and coinfections were not significantly different (19.0% versus 27.3%, *P* value of 0.615 by Chi-squared test).

Overall, the positive subjects responded to 1 to 4 (median, 2) peptides. Among the 13 subjects with cleared infection who showed positive response, five (38.5%) responded to one peptide, three (23.1%) responded to two peptides, four (30.8%) responded to three peptides, and another (7.6%) responded to four peptides. For those with CIN2, two subjects responded to one peptide, one responded to two peptides, two responded to three peptides, and another responded to four peptides. The two subjects with CIN3 responded to only one peptide, while one subject with ICC responded to one peptide, one responded to two, and the other two responded to three peptides.

HLA-A2-specific T-cell epitopes identified from human PBMC. Forty-eight subjects (15 cleared infections, 12 CIN2, 5 CIN3, and 16 ICC) either homologous or heterozygous for the HLA-A2 allele were tested for HPV-58 E6 and E7 peptides. One E6 peptide located at amino acid position 99 to 107 elicited positive responses from two (16.7%) CIN2 subjects with HPV-58 single infection.

HLA-A24-specific T-cell epitopes identified from human PBMC. Thirty-nine subjects (14 cleared infections, 4 CIN2, 9 CIN3, and 12 ICC) either homologous or heterozygous for the HLA-A24 allele were studied. As a result, one L1 peptide located at amino acid position 468 to 476 was found to have a positive response in two (50.0%) CIN2 subjects who had an HPV-58 single infection (Table 2).

DISCUSSION

The strong association between HPV infection and the development of cervical cancer provides an opportunity to tackle

TABLE 6. IFN-γ ELISPOT results according to HLA type and cervical status

HLA	Encoding region, amino acid position[a]	Cervical status	No. with positive ELISPOT result/ no. tested (%)[b]	Mean IFN-γ spot-forming count (range, SD)/10^4 cells	*P* value[c]
A11	L1, 101–109	Cleared infection	7/17 (41.2)	31.7 (18–43, 9.4)	<0.001
		CIN2	0/10 (0)		
		CIN3	0/10 (0)		
		ICC	0/16 (0)		
A11	L1, 296–304	Cleared infection	2/17 (11.8)	32.5 (29–36, 4.9)	1.0
		CIN2	4/10 (40.0)	16.3 (12–21, 4.0)	
		CIN3	0/10 (0)		
		ICC	0/16 (0)		
A11	L1, 327–335	Cleared infection	6/17 (35.3)	35.5 (26–54, 10.4)	<0.001
		CIN2	0/10 (0)		
		CIN3	0/10 (0)		
		ICC	0/16 (0)		
A11	L1, 469–477	Cleared infection	8/17 (47.1)	33.0 (16–45, 10.2)	<0.001
		CIN2	0/10 (0)		
		CIN3	0/10 (0)		
		ICC	0/16 (0)		
A11	E6, 64–72	Cleared infection	1/17 (5.9)	15.0	0.408
		CIN2	2/10 (20.0)	16.0 (15–17, 1.4)	
		CIN3	0/10 (0)		
		ICC	4/16 (25.0)	19.3 (14–25, 4.8)	
A11	E6, 94–102	Cleared infection	0/17 (0)		1.0
		CIN2	2/10 (20.0)	14.7 (11–18, 3.5)	
		CIN3	0/10 (0)		
		ICC	0/16 (0)		
A11	E7, 74–82	Cleared infection	3/17 (17.6)	24.0 (18–30, 6.1)	0.730
		CIN2	4/10 (40.0)	19.8 (12–28, 7.5)	
		CIN3	2/10 (20.0)	14.0 (12–16, 2.8)	
		ICC	3/16 (18.8)	16.0 (13–20, 3.6)	
A11	E7, 78–86	Cleared infection	0/17 (0)		1.0
		CIN2	2/10 (20.0)	13.0 (12–14, 1.4)	
		CIN3	0/10 (0)		
		ICC	0/16 (0)		
A11	E7, 88–96	Cleared infection	0/17 (0)		1.0
		CIN2	0/10 (0)		
		CIN3	0/10 (0)		
		ICC	2/16 (12.5)	13.0 (15–20, 3.5)	
A2	E6, 99–107	Cleared infection	0/15 (0)		1.0
		CIN2	2/12 (16.7)	14.0 (11–17, 4.2)	
		CIN3	0/5 (0)		
		ICC	0/16 (0)		
A24	L1, 468–476	Cleared infection	0/14 (0)		1.0
		CIN2	2/4 (50.0)	15.0 (12–18, 4.3)	
		CIN3	0/9 (0)		
		ICC	0/12 (0)		

[a] Amino acid position numbering is according to the HPV-58 prototype (GenBank accession no. D90400).
[b] Subjects with heterozygous HLA-A alleles were tested for each of the HLA types and, therefore, counted twice.
[c] Positive response rate among subjects with cleared infection compared to that in subjects with CIN2/CIN3/ICC by 2-tailed Fisher's exact test.

cervical cancer by controlling HPV infection (20, 37). The success of prophylactic vaccines has provided a strong impetus to further work in this direction (15, 16, 17, 24). The foreign viral proteins E6 and E7 that are constitutively expressed in tumor cells serve as potential targets for immune clearance and, thus, are the prime targets for therapeutic vaccine development (2, 19, 29, 35). However, it is well documented that lesions that have progressed to a late precancerous or invasive stage often exhibit features of immune escape. The identification of viral epitopes that remain visible to the immune surveillance of women with precancers and invasive cancers is therefore an important step leading to the development of a successful therapeutic vaccine.

Previous studies have mainly focused on HPV-16 and, to a

small extent, HPV-18, the most common HPV types found in cervical cancers worldwide. Earlier studies have shown low positive rates for HPV-16 E6-/E7-specific memory cytotoxic T-cells, ranging from 12% to 20%, among CIN and ICC patients (3, 12, 23, 26). However, at least one recent study using a highly sensitive method has detected an HPV-16 E6-/E7-specific memory cytotoxic-T-cell response in all cervical cancer patients (31). Another study of patients with low-grade squamous intraepithelial lesions showed that while a T-cell response was detected only in half of the patients, the presence of an E2-specific T-cell response correlated with the absence of progression (36). The possibility of augmenting a preexisting, though generally low-level memory cytotoxic-T-cell response leading to the clearance of an established lesion is supported by the results of a recent clinical trial on treating vulvar intraepithelial neoplasia using a synthetic long-peptide vaccine (18).

To date, data on the T-cell response to non-16/-18 high-risk HPV types are not available. We took advantage of having a higher prevalence of HPV-58 in our population to examine the T-cell response to HPV-58 infection. The majority of studies of Western women have focused on a T-cell response restricted by HLA-A*0201 (3, 12). Our study population comprised a large proportion of subjects with HLA-A11 restriction and, therefore, provided some unprecedented data in this area.

In this study, we used an IFN-γ ELISPOT assay to measure the T-cell response because it has the advantage of being less cumbersome than the formal cellular cytotoxicity assay. Although the IFN-γ ELISPOT results correlate with cytotoxicity, one should note that the assay used in this study does not measure the antitumor cytotoxic response directly. Overall, we found that T-cell responses against L1 peptides were mainly observed from subjects who had recently cleared an HPV-58 infection, while T-cell responses specific for E6 and E7 peptides were largely confined to subjects who had developed CIN or ICC. This observation is in line with the expression profile of HPV proteins among different grades of cervical lesion. In cleared infections with normal or low-grade cervical cytological or histological abnormalities, the viral capsid protein L1 is abundantly expressed in the upper, differentiated layer of epithelial cells (11). These proteins may be exposed to antigen-presenting cells with subsequent stimulation of the immune system. The L1-specific T-cell response seems to be short-lived and to disappear once the antigen is no longer expressed, as such response was not observed in patients with CIN3 or above. The current study has identified four potential A11-restricted L1 epitopes, with three of them (amino acid positions 101 to 109, 327 to 335, 469 to 477) showing significantly higher positive rates for subjects who had cleared the infection. It is worth noting that the epitope (L1 296-304) containing a common B-cell epitope (DLYIK) that is present on the surface of virus-like particles also elicited a T-cell response from two subjects with cleared infection and four subjects with CIN2. Although we did not observe a significant difference in response rate between subjects with single and coinfections, the possibility that some of the positive response might due to cross-reactivity with other HPV types could not be excluded. In fact, four of the five L1 epitopes showing a positive response share a high degree of similarity with HPV-16 or HPV-18. It would be interesting to investigate whether the B-cell response also targets epitopes that share a high degree of similarity among different types of HPV and, thus, elicit cross-type immunity following natural infection or vaccination.

Our results showed that T-cell responses to E6 and E7 peptides were only detected infrequently in patients with CIN2 or higher severity disease and, in general, the positive rate among each disease group was less than 20%. This observation may suggest that for subjects who have progressed to CIN2 or higher severity disease, the virus has found a way to escape immune surveillance. Nevertheless, our assay may not be sensitive enough to detect very low levels of T-cell response. Higher response rates have been reported when whole proteins, as opposed to single-epitope-restricted peptides, were used in *in vitro* restimulation (3). Furthermore, an HPV-16 E6-specific memory T-helper response was demonstrated in a substantial proportion of healthy individuals (34), and a lack of cytotoxic response to HPV-16 E6 was also linked to the persistence of infection (22). By examining the T-cell response directly *ex vivo*, the $CD4^+$ but not the $CD8^+$ response was found to be lower among those with progressive disease (30). These findings, together with the results of a recent clinical trial, indicate that there is still a potential to develop therapeutic vaccines to clear established lesions (18).

One limitation of this study is the relatively small number of subjects examined in each disease category. While we attempted to perform statistical analysis on the response rates for each peptide, the type 1 and type 2 statistical errors remained large because of the small sample size. Hence, the statistical significance observed in this study should be taken as a reference for further, more extensive study rather than as final.

We found an E7 epitope (amino acid position 74 to 82) that was broadly reactive across all disease groups. This epitope may be a more immunogenic one, for which even a relatively small amount of E7 protein produced during transient infection is sufficient to elicit a T-cell response. Since the response rate to this epitope was similar across all disease groups, it does not seem to play a protective role. We have also identified two E6 peptides (amino acid positions 64 to 72 and 94 to 102), three A11-restricted E7 peptides (amino acid positions 74 to 82, 78 to 86, and 88 to 96), and one A2-restricted E6 peptide (99 to 107) for which a natural T-cell response could be developed. Nevertheless, it is not known whether these HPV peptides are naturally processed and presented in cervical epithelial cells. Furthermore, our results only suggest that these epitopes are immunogenic. Further study is required to verify whether these epitopes can elicit a protective immune response.

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