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Detection of Human Papillomavirus Type 16-Specific T Lymphocytes by a Recombinant Vaccinia Virus-Based Enzyme-Linked Immunospot Assay▽

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Cell-mediated immunity, particularly that induced by T cells, is thought to have a key role in controlling infection. The enzyme-linked immunospot (ELISPOT) assay has been successfully adapted to detect T-cell immune response to a variety of pathogens. However, it still remains a challenge to detect antigen-specific T cells when the numbers of circulating cells are low, such as in a local cervical infection caused by genital human papillomavirus (HPV). The goal of this study was to develop a protocol for enhanced detection of HPV-specific CD8$^+$ T cells by examining a number of the variables involved in performing an ELISPOT assay. Since blood samples consistently positive for HPV-specific T cells are difficult to obtain, previously described human papillomavirus type 16 (HPV16) E6 52-61 (FAFRDLCIVY)-specific T-cell clone cells (13) seeded in peripheral blood mononuclear cells from an HLA-B57-positive blood donor were used. The variables examined were the amounts of primary and secondary anti-gamma interferon antibodies, amounts of antigen-presenting monocytes and recombinant vaccinia virus expressing the HPV16 E6 protein, and amounts of exogenous cytokines added (recombinant human interleukin-2 [rhIL-2] and rhIL-7). The amounts of antigen-presenting monocytes, followed by the concentration of exogenous rhIL-2, had the most pronounced and significant effects in enhancing sensitivity of the ELISPOT assay. Blood samples from six patients being monitored for abnormal Pap smear results and from 12 healthy volunteers were examined using the enhanced conditions.

The enzyme-linked immunospot (ELISPOT) assay is a sensitive and quantitative assay that has been widely used to detect antigen-specific cytokine-secreting T cells at the single-cell level. Although the ELISPOT assay has been shown to be highly sensitive and reproducible (19), detecting low-frequency circulating memory T cells specific for nonsystemic viral antigens such as human papillomavirus (HPV) without in vitro expansion remains a challenge. Larsson et al. have successfully used the recombinant vaccinia virus-based ELISPOT assay to detect low frequency human immunodeficiency virus (HIV)-specific T cells in peripheral blood (10, 11; see also reference 3). Recently, Farhat et al. have also adapted the recombinant vaccinia virus-based ELISPOT assay and were able to detect low-frequency circulating HPV-specific T cells in the peripheral blood of women with persistent HPV type 16 (HPV16) infection and of those who have cleared their infection (S. Farhat, M. Nakagawa, K. H. Kim, and A.-B. Moscicki, unpublished data). Our goal for this study was to further enhance the detection of HPV-specific T cells in peripheral blood by performing rigorous analysis of various assay conditions.

The recombinant vaccinia virus-based ELISPOT assay has several desirable characteristics. It circumvents the need for prior knowledge of the T-cell epitope and the corresponding restricting major histocompatibility complex class I (MHC-I) element. Other advantages of using the recombinant vaccinia virus are that it infects a broad range of mammalian antigen-presenting cells (APC) and efficiently synthesizes relatively high levels of recombinant gene products that are endogenously processed and presented via the MHC-I pathway to CD8$^+$ T cells.

In order to optimize the sensitivity of our recombinant vaccinia virus-based ELISPOT assay, we made use of human CD8$^+$ T-cell clone cells specific for HPV16 E6 52-61 (FAF RDLCIVY) restricted by HLA-B57 isolated in our laboratory. We examined the effects of adjusting (i) the concentrations of primary and secondary anti-gamma interferon (IFN-γ) monoclonal antibodies, (ii) the number of APCs (HLA-B57 matched allogeneic monocytes infected with recombinant vaccinia virus expressing HPV16 E6 protein [E6-vac]), (iii) the multiplicity of infection (MOI) of E6-vac, and (iv) the concentrations of exogenous recombinant human interleukin-2 (rhIL-2) and rhIL-7. Blood samples from patients being monitored for abnormal Pap smear results and healthy volunteers were analyzed under the conditions determined using the T-cell clone.

MATERIALS AND METHODS

Cells. Human CD8$^+$ T-cell clone cells specific for HPV16 E6 52-61 (FAFRD LCIVY) restricted by HLA-B57 were used as the antigen-specific IFN-γ-secreting cells (13). Briefly, CD8$^+$ T cells from a woman who had acquired and cleared HPV16 infection as detected by PCR analysis of cervical samples were stimulated with autologous mature dendritic cells infected with recombinant vaccinia viruses expressing the HPV16 E6 and E7 proteins. The presence of HPV-specific T cells was demonstrated using an IFN-γ ELISPOT assay with a series of pooled

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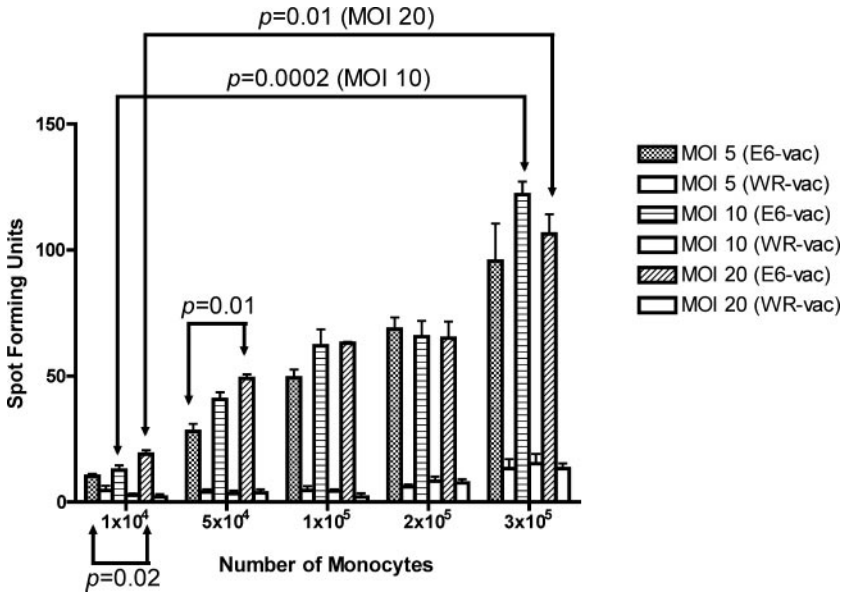

FIG. 1. ELISPOT assay determining the effects of increasing the number of E6-vac-infected HLA-B57-positive monocytes and the MOI for E6-vac. The adjacent white bars represent the corresponding WR-vac-negative controls. The numbers of SFU increased as more monocytes were added. The differences between the results seen with 1×10^4 monocytes and with 3×10^5 monocytes were statistically significant at an MOI of 10 ($P = 0.0002$) and an MOI of 20 ($P = 0.01$). Furthermore, at smaller numbers of monocytes (1×10^4 and 5×10^4), the increases in MOI from an MOI of 5 to an MOI of 20 were statistically significant at 1×10^4 ($P = 0.02$) and at 5×10^4 ($P = 0.01$). The error bars represent standard errors of the means. Results representative of three experiments are shown.

overlapping 15-mer peptides covering the entire length of the E6 and E7 proteins. The strongest response was seen in the E6 46 to 70 region, and the specific T-cell clones were selected magnetically on the basis of IFN-γ secretion. Multiple clones were isolated and characterized and were found to have the same characteristics. Nevertheless, clone 2 was used for this study for consistency and was expanded on a feeder cell mixture (Yssel's medium [Gemini Bioproducts, Inc., Calabasas, CA] containing 1% pooled human serum, penicillin G [100 U/ml], streptomycin [100 μg/ml], 1×10^6/ml irradiated allogeneic peripheral blood mononuclear cells [PBMC], 1×10^5/ml irradiated JY cells, and 0.1 μg/ml phytohemagglutinin [PHA]).

HLA-B57 matched allogeneic monocytes were obtained from buffy coat units drawn from HLA-B57-positive donors (Lifeblood Mid-South Regional Blood Center, Memphis, TN). PBMC were isolated from the buffy coat by density gradient centrifugation using Ficoll-Paque PLUS (Amersham Biosciences, Piscataway, NJ). $CD14^+$ monocytes were positively selected using MACS CD14 MicroBeads and LS columns according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA).

Subjects. The patients being monitored for abnormal Pap smear results and who were therefore likely to have HPV infection or have had HPV infection were recruited from the University of Arkansas for Medical Sciences Obstetrics and Gynecology clinics. Healthy volunteers were recruited among students and hospital and research laboratory workers at the same institution. The protocols were approved by the University of Arkansas for Medical Sciences internal review board, and informed consent was obtained from each participant.

IFN-γ ELISPOT assay. A method described by Larsson and colleagues was used with minor modifications (10). Unless otherwise mentioned, the following standard conditions were used. Briefly, 96-well plates (MultiScreen-HA; Millipore, Bedford, MA) were coated with 5 μg/ml of primary anti-IFN-γ monoclonal antibody (Mabtech, Stockholm, Sweden) for capture in 50 μl/well of phosphate-buffered saline (PBS) and stored at 4°C overnight. The plates were then washed four times with PBS and blocked using RPMI 1640–5% pooled human serum for 1 h at 37°C.

$CD8^+$ T-cell clone cells were plated at 200 cells per well. Monocytes from HLA-B57-positive individuals were infected with recombinant vaccinia virus expressing HPV16 E6 protein or a WR-parental vaccinia virus (WR-vac) strain at a MOI of 10 for 1 h at 37°C prior to being plated at 1×10^5 cells/well. CD14-depleted PBMC (100,000) from the same HLA-B57 matched buffy coat and rhIL-2 (R&D Systems, Inc., Minneapolis, MN), at a final concentration of 20 U/ml, were also added. PHA was added at 10 μg/ml to positive-control wells.

After a 20-h incubation at 37°C, the plate was washed four times with PBS–0.05% Tween 20. A total of 50 μl of secondary antibody (enzyme labeled for detection) in PBS at a final concentration of 1 μg/ml biotin-conjugated anti-IFN-γ monoclonal antibody (Mabtech) was added, and the plate was incubated for 2 h at 37°C. The plate was then washed four times with PBS–0.1% Tween 20. Avidin-bound biotinylated horseradish peroxidase H (Vectastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA) was added for 1 h at 37°C. After four washings with PBS–0.1% Tween 20, stable diaminobenzene (Open Biosystems, Huntsville, AL) was added to develop the reaction. The plates were washed with distilled water three times and air dried overnight. Spot-forming units (SFU) were counted using an automated ELISPOT analyzer (Cell Technology, Inc., Jessup, MD). Net SFU were calculated by subtracting the number of SFU from the corresponding WR-vac well from the number of SFU from the E6-vac well.

Primary and secondary anti-IFN-γ antibodies. In order to determine the optimal concentrations of primary and secondary anti-IFN-γ monoclonal antibodies, plates were coated with increasing concentrations of 5, 10, 15, or 20 μg/ml of primary anti-IFN-γ monoclonal antibody and 1 or 5 μg/ml secondary biotin-conjugated anti-IFN-γ monoclonal antibody. In experiments in which the E6 52-61 peptide (10 μM) was used as the antigen, cells from an autologous Epstein-Barr virus-transformed lymphoblastoid cell line (1×10^5/well) served as APC. Net SFU values were calculated by subtracting the number of SFU of the corresponding well without peptide from the number of SFU of the well with peptide.

Number of monocytes and multiplicity of E6-vac infection. Recombinant vaccinia virus expressing the entire E6 protein was used as the source of antigen. HLA-B57 matched allogeneic monocytes were infected with E6-vac or WR-vac at a MOI of 5, 10, or 20. The number of infected monocytes per well was also adjusted to 1×10^4, 5×10^4, 1×10^5, 2×10^5, or 3×10^5 per well (Fig. 1). The effect of increasing numbers of monocytes and MOI was calculated as described above for primary and secondary antibodies.

rhIL-2 and rhIL-7. The concentrations of exogenously added cytokines were also adjusted: rhIL-2 at 0 U/ml, 10 U/ml, 20 U/ml, or 30 U/ml and rhIL-7 (R&D Systems, Inc.) at 0 ng/ml, 5 ng/ml, 10 ng/ml, 15 ng/ml, 20 ng/ml, or 25 ng/ml (Fig. 2). The effect of increasing concentrations of rhIL-2 and rhIL-7 was calculated as described above for primary and secondary antibodies.

Serial dilution of HPV-specific T-cell clone cells. The HPV16 E6 52-61-specific $CD8^+$ T-cell clone cells were serially diluted by a factor of 2 starting from 1,000 cells for a total of eight times. Monocytes from an HLA-B57-positive individual were infected with E6-vac or WR-vac at MOI of 10 for 1 h at 37°C prior to being

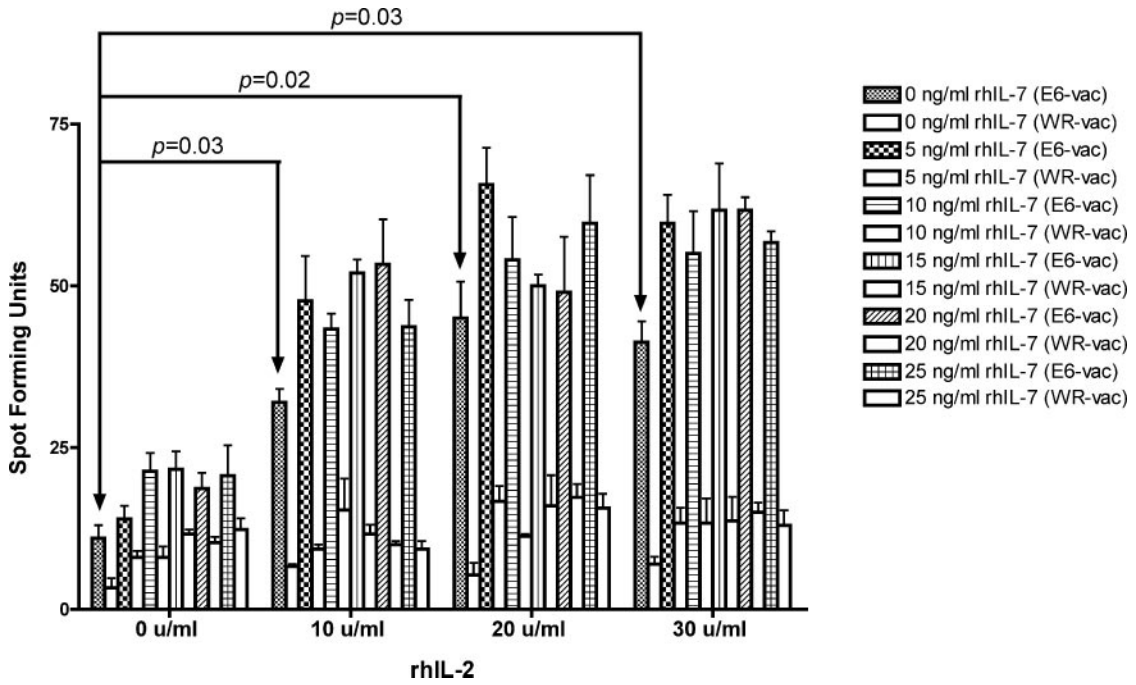

FIG. 2. ELISPOT assay evaluating the effects of adding exogenous rhIL-2 and rhIL-7. The adjacent white bars are the corresponding WR-vac-negative controls. Statistically significant increases in the numbers of SFU were demonstrated when rhIL-2 was added alone compared to the results seen in the absence of rhIL-2 and rhIL-7 ($P = 0.03$ at 10 units/ml, $P = 0.02$ at 20 units/ml, and $P = 0.03$ at 30 units/ml). However, the addition of rhIL-7 alone did not yield statistically significant results. Interestingly, the addition of both rhIL-2 and rhIL-7 may have an additive effect although the effect may be only very slightly above that seen with rhIL-2 alone. The error bars represent standard errors of the means. Results representative of two experiments are shown.

plated at 1×10^5/well or 2×10^5/well. Otherwise, the standard conditions described above were used for the ELISPOT assy. Net SFU values were calculated by subtracting the number of SFU of the corresponding WR-vac negative-control well from the number of SFU of the E6-vac well.

Examining human subject samples using the enhanced conditions. Whole blood (40 to 80 ml) was collected in Vacutainer tubes containing sodium heparin. PBMC were isolated as described above from the buffy coat units, and ELISPOT assays were performed using 5 μg/ml of primary anti-IFN-γ monoclonal antibody, 1 μg/ml secondary biotin-conjugated anti-IFN-γ monoclonal antibody, and 3×10^5 monocytes per well infected with recombinant vaccinia viruses (E6-vac, E7-vac, and WR-vac) at an MOI of 5 in triplicate wells. Monocyte-depleted PBMC (100,000 per well) and 20 units/ml of exogenous rhIL-2 were added. These were the conditions we believed to be optimal both for the T-cell detection (Fig. 1 and 2) and for the use of laboratory resources. In separate wells, PHA was added (10 μg/ml) as a positive control. The results were considered positive when the averaged SFU numbers in the experimental wells were more than twice those of the negative-control (WR-vac) wells (7, 14).

Statistical analysis. The *t* test for paired samples was used to determine the statistical significance of the numbers of IFN-γ-secreting T cells detected under the various experimental conditions. All *P* values were two-tailed, and the significance level was set at 0.05. The analysis was performed using InStat Version 3.0 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Effects of adjusting the amounts of primary and secondary anti-IFN-γ antibody. The effects of the different combinations of primary and secondary anti-IFN-γ antibody concentrations were examined. The determination of the range of concentrations of primary and secondary anti-IFN-γ antibodies tested was based on the amounts used by Larsson et al. (5 μg/ml and 1 μg/ml, respectively) and Farhat et al. (20 μg/ml and 4 μg/ml, respectively) (10). The numbers of SFU did not differ significantly when the concentration of the primary antibody (5 μg/ml, 10 μg/ml, 15 μg/ml, and 20 μg/ml) or the secondary antibody (1 μg/ml and 5 μg/ml) was adjusted (data not shown). At a secondary antibody concentration of 1 μg/ml, the primary antibody was adjusted to 5 μg/ml, 10 μg/ml, 15 μg/ml, and 20 μg/ml; the net SFU were 53.0 ± 9.2, 63.7 ± 2.1, 73.0 ± 1.6, and 65.7 ± 5.2, respectively. Similarly, the net SFU were 68.0 ± 2.9, 68.0 ± 5.0, 64.3 ± 3.1 and 58.3 ± 7.1, respectively, at a secondary antibody concentration of 5 μg/ml. There was no enhancement in sensitivity observed as a result of adjusting the concentrations of the anti-IFN-γ antibodies. Similar results were obtained when the E6 52-61 peptide was used as the antigen (data not shown).

Effects of adjusting the number of monocytes and multiplicity of E6-vac infection. The numbers of E6-vac-infected HLA-B57-positive monocytes and the MOI for E6-vac were adjusted (Fig. 1). At an MOI of 5, increasing the number of monocytes (1×10^4, 5×10^4, 1×10^5, 2×10^5, and 3×10^5) increased the number of net SFU (5.7 ± 4.7, 24.0 ± 5.0, 44.7 ± 8.5, 62.7 ± 9.8, and 82.3 ± 30.1, respectively). Similar results were obtained at an MOI of 10 (10.0 ± 2.6, 37.3 ± 6.4, 57.7 ± 10.1, 57.3 ± 12.5, and 106.7 ± 3.1, respectively) and at an MOI of 20 (17.0 ± 3.6, 45.3 ± 1.5, 61.0 ± 2.6, 57.3 ± 9.5, and 93.0 ± 15.1, respectively). The differences between the numbers of monocytes (1×10^4 and 3×10^5) were statistically significant at MOIs of 10 and 20 ($P = 0.0002$ and $P = 0.01$, respectively).

On the other hand, a significant effect of increasing MOI (5, 10, and 20) was observed with smaller numbers of monocytes (1×10^4 and 5×10^4) but not with the larger numbers ($1 \times$

10^5, 2×10^5, and 3×10^5). The *P* values seen at MOIs between 5 and 20 were $P = 0.02$ at 1×10^4, $P = 0.01$ at 5×10^4, $P = 0.12$ at 1×10^5, $P = 0.57$ at 2×10^5, and $P = 0.71$ at 3×10^5.

Effects of adjusting the concentrations of rhIL-2 and rhIL-7. The effects of adding various concentrations of exogenous rhIL-2 (0 U/ml, 10 U/ml, 20 U/ml, and 30 U/ml) and rhIL-7 (0 ng/ml, 5 ng/ml, 10 ng/ml, 15 ng/ml, 20 mg/ml, and 25 ng/ml) were determined (Fig. 2). When no rhIL-7 was present, the net SFU were 7.7 ± 4.0, 25.3 ± 3.8, 39.7 ± 7.5, and 34.3 ± 6.8 as the concentration of rhIL-2 was increased. Addition of rhIL-2 at all concentrations significantly increased the number of detected IFN-γ-secreting cells relative to those seen in the absence of rhIL-2 and rhIL-7 ($P = 0.03$ at 10 units/ml, $P = 0.02$ at 20 units/ml, and $P = 0.03$ at 30 units/ml). However, the addition of rhIL-7, in the absence of rhIL-2, did not show any enhancement in detection. When no rhIL-2 was present, the net SFU were 7.7 ± 4.0, 6.0 ± 3.0, 13.0 ± 6.0, 10.0 ± 5.6, 8.3 ± 5.5, and 8.3 ± 11.0 at increasing concentrations of rhIL-7. Increases in the detection of background SFU were noticed as the concentration of rhIL-7 was increased, but the differences were not statistically significant.

When both rhIL-2 and rhIL-7 were added, there was some suggestion of an additive effect. When the numbers of SFU for the 15 different combinations of rhIL-2 and rhIL-7 were compared to the results seen in the absence of rhIL-2 and rhIL-7, 11 combinations were statistically significant: 10 U/ml rhIL-2 and 5 ng/ml rhIL-7 ($P = 0.035$), 10 U/ml rhIL-2 and 10 ng/ml rhIL-7 ($P = 0.031$), 10 U/ml rhIL-2 and 15 ng/ml rhIL-7 ($P = 0.011$), 10 U/ml rhIL-2 and 20 ng/ml rhIL-7 ($P = 0.043$), 20 U/ml rhIL-2 and 5 ng/ml rhIL-7 ($P = 0.008$), 20 U/ml rhIL-7 and 10 ng/ml rhIL-7 ($P = 0.019$), 20 U/ml rhIL-2 and 25 ng/ml rhIL-7 ($P = 0.008$), 30 U/ml rhIL-2 and 5 ng/ml rhIL-7 ($P = 0.022$), 30 U/ml rhIL-2 and 15 ng/ml rhIL-7 ($P = 0.042$), 30 U/ml rhIL-2 and 20 ng/ml rhIL-7 ($P = 0.019$), and 30 U/ml rhIL-2 and 25 ng/ml rhIL-7 ($P = 0.001$). In order to rule out the possibility of the apparent additive effect being solely contributed by rhIL-2, a comparison was made between the effect of rhIL-2 alone (the ratio between the results seen with rhIL-2 alone and those seen in the absence of both rhIL-2 and rhIL-7) and the different combinations of rhIL-2 and rhIL-7 (the ratio between the results seen with rhIL-2 combined with rhIL-7 and those seen in the absence of both rhIL-2 and rhIL-7). The same 11 combinations that demonstrated statistical significance showed increased numbers of SFU with rhIL-2 combined with rhIL-7 compared to the results seen with rhIL-2 alone: 10 U/ml rhIL-2 and 5 ng/ml rhIL-7 (25.3 ± 3.8 to 38.3 ± 11.0), 10 U/ml rhIL-2 and 10 ng/ml rhIL-7 (25.3 ± 3.8 to 28.0 ± 9.8), 10 U/ml rhIL-2 and 15 ng/ml rhIL-7 (25.3 ± 3.8 to 40.3 ± 2.3), 10 U/ml rhIL-2 and 20 ng/ml rhIL-7 (25.3 ± 3.8 to 43.3 ± 11.6), 20 U/ml rhIL-2 and 5 ng/ml rhIL-7 (39.7 ± 7.5 to 49.0 ± 8), 20 U/ml rhIL-7 and 10 ng/ml rhIL-7 (39.7 ± 7.5 to 42.7 ± 11.7), 20 U/ml rhIL-2 and 25 ng/ml rhIL-7 (39.7 ± 7.5 to 44.0 ± 9.5), 30 U/ml rhIL-2 and 5 ng/ml rhIL-7 (34.3 ± 6.8 to 46.3 ± 6.1), 30 U/ml rhIL-2 and 15 ng/ml rhIL-7 (34.3 ± 6.8 to 48.0 ± 13.9), 30 U/ml rhIL-2 and 20 ng/ml rhIL-7 (34.3 ± 6.8 to 46.7 ± 5.7), and 30 U/ml rhIL-2 and 25 ng/ml rhIL-7 (34.3 ± 6.8 to 44.0 ± 4.6). Therefore, the addition of both rhIL-2 and rhIL-7 may have an additive effect, although this effect may be only very slightly above that seen with rhIL-2 alone.

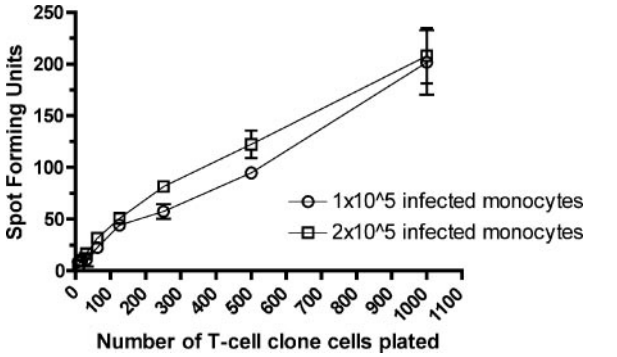

FIG. 3. Serial dilution of HPV-specific $CD8^+$ T-cell clone cells. The E6 52-61-specific $CD8^+$ T-cell clone cells were serially diluted 8 times by a factor of 2 starting from 1,000 cells. Infected monocytes were plated at either 1×10^5 per well or 2×10^5 per well. The correlations between the numbers of T-cell clone cells added and the numbers of SFU detected were nearly linear. The error bars represent standard errors of the means.

Serial dilution of HPV-specific $CD8^+$ T-cell clone cells. One limitation of using T-cell clone cells as a source of HPV-specific T cells is the difficulty in obtaining accurate cell counts. Because these T cells are not transformed, they require the presence of irradiated feeder cells for continuous growth in culture. Therefore, when cell counts are determined by trypan blue exclusion using a hemocytometer, both HPV-specific T cells and irradiated but still living cells from allogeneic PBMC are included. We believe this is the major reason that the SFU values shown in Fig. 1 and 2 are less than 200. In order to ascertain that the detectability of HPV-specific T cells does not decrease when few HPV-specific T cells are present, a serial dilution of the E6 52-61-specific $CD8^+$ T-cell clone cells was performed. This was followed by performance of a recombinant vaccinia virus-based ELISPOT assay under standard conditions (see Materials and Methods) (Fig. 3). E6-vac-infected monocytes (100,000 and 2×10^5) were added as antigen-presenting cells. As shown in Fig. 3, nearly linear correlations between the number of T-cell clone cells added and the number of SFU detected have been shown under both sets of conditions, although only about a quarter of the total number of cells plated resulted in SFU. These results demonstrate that the recombinant vaccinia virus-based ELISPOT assay is capable of detecting HPV-specific T cells even when their numbers are extremely low.

Examining human subject samples under the enhanced conditions. Blood samples from six patients being followed for abnormal Pap smear results, and 12 healthy volunteers were analyzed. The age, sex, clinical profiles, and the ELISPOT results for the subjects are summarized in Table 1. Two of the six clinic patients were positive for high-risk HPV types as determined by Digene's Hybrid Capture II test, and the remaining patients were not tested as a part of their routine medical care. All patients except for subject 1 were treated using cryotherapy (subject 3) or a loop electrical excision procedure (subjects 2, 4, 5, and 6). At the time of the blood draw, two patients had negative Pap smear results (subjects 1 and 3)

TABLE 1. Subject age, sex, pap smear, biopsy, and ELISPOT results[a]

Subject	Category	Age (yr)	Sex	Initial Pap result(s)	Biopsy result	Treatment	Pap result at blood draw	ELISPOT result[b]	
								E6-vac	E7-vac
1	Patient	45	F	ASCUS, HPV$^+$	ND	None	−	630	607
2	Patient	31	F	HSIL	CIN1	LEEP	LSIL	180	−
3	Patient	21	F	LSIL	ND	Cryotherapy	−	−	−
4	Patient	37	F	ASCUS, HPV$^+$	CIN1	LEEP	LSIL	−	−
5	Patient	29	F	ASCUS × 2	CIN2/3	LEEP	HSIL	−	−
6	Patient	27	F	HSIL	CIN1	LEEP	ASCUS	−	−
7	Healthy	40	F	NA	NA	NA	NA	−	−
8	Healthy	38	F	NA	NA	NA	NA	−	−
9	Healthy	34	M	NA	NA	NA	NA	−	−
10	Healthy	40	F	NA	NA	NA	NA	−	−
11	Healthy	24	M	NA	NA	NA	NA	−	−
12	Healthy	23	M	NA	NA	NA	NA	−	−
13	Healthy	49	F	NA	NA	NA	NA	−	−
14	Healthy	41	F	NA	NA	NA	NA	−	−
15	Healthy	41	M	NA	NA	NA	NA	−	−
16	Healthy	45	M	NA	NA	NA	NA	−	−
17	Healthy	45	F	NA	NA	NA	NA	197	107
18	Healthy	52	F	NA	NA	NA	NA	−	−

[a] F, female; M, male; ASCUS, atypical cells of undetermined significance; ND, not done; HSIL, high-grade squamous intraepithelial lesion; CIN1 and CIN2, cervical intraepithelial neoplasia grades 1 and 2; NA, not applicable; LEEP, loop electrosurgical excision procedure; LSIL, low-grade squamous epithelial lesion. −, negative result.

[b] Numbers of spot-forming units per million PBMC after subtracting the number calculated for the background to WR-vac in samples with positive results.

whereas the other patients had abnormal results ranging from atypical squamous cells of undetermined significance to high-grade squamous intraepithelial lesions. As for the ELISPOT results, two of six (33%) patients and 1 of 12 (8%) healthy volunteers were positive for the HPV16 E6 protein whereas one of six (17%) patients and 1 of 12 (8%) healthy volunteers were positive for the E7 protein.

DISCUSSION

Traditionally, chromium release assays were used to study the function of CD8$^+$ T lymphocytes by measuring the amount of chromium released in the media. This method had a number of disadvantages, which included being labor intensive, requiring the use of radioisotope and large number of cells, and the results being semiquantitative (i.e., they do not yield information on the quantity of antigen-specific T cells). Recent advances in immunological methods have given birth to a number of methods which can measure the T-cell response, at least in theory, at the single-cell level, including the ELISPOT assay (19), cytokine flow cytometry (6, 16) and tetramer analysis (1). Tetramer analysis is limited to studying peptide antigens, and therefore can only be used to study populations with certain HLA types. The ELISPOT assay and cytokine flow cytometry have the flexibility of being able to use a variety of antigen types, which include peptides, whole proteins, proteins expressed by recombinant viruses, and whole viruses. When working with a pathogen such as HPV, which cannot readily be grown in culture in sufficient quantities, one must use other forms of antigen. The use of proteins produced by recombinant viruses is particularly advantageous, because the antigen is processed by the endogenous protein synthetic machinery of the host cells, assuring that the antigen is processed naturally. In addition, subjects of all HLA types can be studied. Another advantage of the more recently developed methods compared to the traditional chromium release assay is that they can often be used for direct ex vivo quantification of antigen-specific T cells whereas the chromium release assay usually requires in vitro stimulation.

Studying cell-mediated immune responses to HPV has been our long-term research interest. Even with the recently developed sensitive methods, measuring HPV-specific T cells in periphery is a challenge because of low numbers of circulating HPV-specific T cells. The goal of this study was to optimize the recombinant vaccinia virus-based ELISPOT assay by looking at a number of specific variables in order to enhance detection. As a reliable source of HPV-specific T cells, a small number of human CD8$^+$ T-cell clone cells previously isolated (13) was used to seed CD14-depleted PBMC. To our knowledge, this is the first attempt to optimize the ELISPOT assay using a human CD8$^+$ T-cell clone, although work using a murine CD8$^+$ T-cell clone specific for a rodent malaria epitope has been reported previously (12).

Among the variables examined (concentrations of primary and secondary anti-IFN-γ antibodies, numbers of APC and MOI, and concentrations of exogenous rhIL-2 and rhIL-7), increasing the numbers of APC showed the most pronounced effect in enhancing detection. Buseyne et al. (3) have shown that CD14$^+$-CD15$^+$ monocytes were the main cells presenting antigens expressed by recombinant vaccinia viruses, and others have also shown that higher concentrations of APC can lead to enhanced detection of T cells. One group of investigators showed that detection was significantly better with 1×10^5 APC (MHC-I-expressing P815 mastocytoma cell line) compared to the results seen with 1×10^3, 1×10^4, or 1×10^6 APC when 20 to 130 T-cell clone cells were added per well (12). In our study, the maximum amount of monocytes added was 3×10^5 per well, the quantity at which the largest number of SFU were detected. We have not determined whether further increasing the numbers of APC will enhance detection or

whether a decrease or plateau in the number of SFU will be observed. However, from a practical point of view, further increasing the numbers of monocytes may require drawing a larger volume of blood, and this may not always be feasible. Nevertheless, our data suggest that it is important to use a relatively large number of monocytes for sufficient detection of the antigen-specific T cells.

Schmittel et al. studied the application of the IFN-γ ELISPOT assay to quantify T-cell responses to proteins such as tetanus toxoid and purified-protein derivative and concluded that the APC concentration was one of the key determinants in enhanced sensitivity of T-cell detection (18). Interestingly, their experiments revealed increased enumeration of tetanus toxoid- or purified-protein derivative-reactive $CD4^+$ T lymphocytes as the concentration of protein-presenting monocytes was increased from 4% to 16% but not of 9-mer peptide (from the influenza matrix protein)-reactive $CD8^+$ T lymphocytes. Our study showed enhanced detection of $CD8^+$ T lymphocytes as the amount of E6-vac-infected monocytes was increased. It seems plausible that detection is enhanced as the number of monocytes is increased in cases in which the antigen, such as a whole protein or protein expressed by recombinant vaccinia virus, requires processing but not when a fully processed antigen such as a 9-mer peptide is used.

The use of exogenous rhIL-2 to improve detection of antigen-specific T cells is prevalent (3, 12, 13, 15, 17), and enhanced T-cell proliferation (9) and detection (5) have also been reported for rhIL-7. In this study, enhanced detection of antigen-specific $CD8^+$ T cells was shown when rhIL-2 was added but not for rhIL-7 alone. Moreover, this effect by rhIL-2 seemed to plateau, and the enhancement was almost the same at 20 U/ml and 30 U/ml. Interestingly, the addition of rhIL-2 and rhIL-7 together may have an additive effect although the effect may be only very slightly above that seen with rhIL-2 alone.

Increasing the primary and secondary anti-IFN-γ antibody concentrations did not enhance detection, suggesting that saturating amounts of antibody are present under the standard conditions (10). Doubling and quadrupling MOI enhanced detection significantly when low numbers of monocytes were used but not when larger numbers were present. These observations can be interpreted in this way: the numbers of APC presenting the specific peptide antigen can be increased either by increasing the number of monocytes or by increasing the MOI until reaching the point at which numbers of APC are present that are saturating with respect to the number of T-cell clone cells present.

Because blood samples consistently positive for the presence of HPV-specific T cells are difficult to obtain, we utilized an HPV16 E6 52-61-specific $CD8^+$ T-cell clone to examine various ELISPOT assay conditions. Although a $CD8^+$ T-cell clone was used, the ELISPOT assay using PBMC from human subjects may detect $CD4^+$ as well as $CD8^+$ T lymphocytes, since both are capable of secreting IFN-γ upon stimulation by the antigen. Other groups have also utilized an IFN-γ ELISPOT assay to measure the numbers of HPV16-specific T cells. Synthetic peptides were used as sources of antigen for the HPV16 E6 and E7 protein, and CD4 and CD8 T lymphocytes were isolated and analyzed separately using blood samples from patients with cervical intraepithelial neoplasia and cervical cancer (20). This group reported very prevalent CD8 responses to the HPV16 E6 peptides across all disease grades, but our results showed positive results for the E6-vac-infected targets for only two of six (33%) of the patients. It would be difficult to draw any conclusions from comparisons of these studies, since there are significant differences in the methodologies and patients' clinical conditions. The patients in the other study were untreated at the time of the blood draw. Welters and colleagues (21) employed an IFN-γ ELISPOT assay after 4 days of in vitro stimulation using the HPV16 E6 peptides to examine healthy blood donors and demonstrated positive responses to at least one of the peptides in 12 of 20 (60%) of them. In our study, only 2 of 12 (17%) of the healthy donors demonstrated positive responses to E6. Again, it would be difficult to draw any conclusions in comparisons of these studies due to the differences in the methodologies and the subject population, but it may be possible that the positive response to E6 is lower in our healthy donor group because the ex vivo ELISPOT we perform may only detect effector lymphocytes while addition of in vitro stimulation may result in the detection of memory lymphocytes (2, 8). Another group has reported a more prevalent detection of HIV-specific T lymphocytes with larger numbers of SFU when a cultured ELISPOT assay (which includes in vitro stimulation) was used compared to the results obtained with an ex vivo ELISPOT assay in a side-by-side comparison of results seen with recipients of HIV vaccine (4). The main goal of this study was to determine the optimal conditions for detection of HPV16-specific T lymphocytes through the use of the ex vivo IFN-γ ELISPOT assay. In short, we have demonstrated that key variables in significantly enhancing detection of IFN-γ-secreting $CD8^+$ T cells are large numbers of APC and addition of exogenous rhIL-2.

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