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

Kevin H. Kim,1* William Greenfield,2 Ezekiel Shotts,1 and Mayumi Nakagawa1,3

Departments of Dermatology,1 Obstetrics and Gynecology,2 and Pathology,3 University of Arkansas for Medical Sciences, Little Rock, Arkansas

Received 7 December 2006/Returned for modification 22 January 2007/Accepted 8 February 2007

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 (FAFRDLCIVY) 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 (FAFRDLCIVY) 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

* Corresponding author. Mailing address: Department of Dermatology, School of Medicine, University of Arkansas for Medical Sciences, 4301 West Markham Street, Slot 576, Little Rock, AR 72205. Phone: (501) 686-8635. Fax: (501) 686-7264. E-mail: mnakagawa@uams.edu.

Published ahead of print on 21 February 2007.
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 × 10^4 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 × 10^3 and 5 × 10^3), 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.
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 × 10^5 and 3 × 10^5).
The effects of adjusting the concentrations of rhIL-2 and rhIL-7.

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 < 0.035). 10 U/ml rhIL-2 and 5 ng/ml rhIL-7 (P = 0.031), 10 U/ml rhIL-2 and 10 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-2 and 10 ng/ml rhIL-7 (P = 0.019), 20 U/ml rhIL-2 and 25 mg/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 results seen with rhIL-2 alone and those seen in the absence of 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-2 and 10 ng/ml rhIL-7 (39.7 ± 7.5 to 42.7 ± 11.7), 20 U/ml rhIL-2 and 25 mg/ml rhIL-7 (39.7 ± 7.5 to 44.9 ± 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 mg/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.

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 × 10⁵) 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).
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 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 vitro stimulation. Cell-based assays for primary screening, such as chromium release assays, benefit from the use of antigen-presenting cells (APC) to process and present the antigen to 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-\(\gamma\) 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 \(\times\) 10\(^{5}\) APC (MHC-I-expressing P815 mastocytoma cell line) compared to the results seen with 1 \(\times\) 10\(^{3}\), 1 \(\times\) 10\(^{4}\), or 1 \(\times\) 10\(^{5}\) 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 \(\times\) 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 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.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Category</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Initial Pap result(s)</th>
<th>Biopsy result</th>
<th>Treatment</th>
<th>Pap result at blood draw</th>
<th>ELISPOT result (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Patient</td>
<td>45 F</td>
<td>ASCUS, HPV(^+)</td>
<td>ND</td>
<td>None</td>
<td>-</td>
<td>630</td>
<td>607</td>
<td></td>
</tr>
<tr>
<td>2 Patient</td>
<td>31 F</td>
<td>HSIL</td>
<td>CIN1</td>
<td>LEEP</td>
<td>LSIL</td>
<td>180</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3 Patient</td>
<td>21 F</td>
<td>LSIL</td>
<td>ND</td>
<td>Cryotherapy</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4 Patient</td>
<td>37 F</td>
<td>ASCUS, HPV(^+)</td>
<td>CIN1</td>
<td>LEEP</td>
<td>LSIL</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5 Patient</td>
<td>29 F</td>
<td>ASCUS (\times) 2</td>
<td>CIN2/3</td>
<td>LEEP</td>
<td>HSIL</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6 Patient</td>
<td>27 F</td>
<td>HSIL</td>
<td>CIN1</td>
<td>LEEP</td>
<td>ASCUS</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>7 Healthy</td>
<td>40 F</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>8 Healthy</td>
<td>38 F</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>9 Healthy</td>
<td>34 M</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>10 Healthy</td>
<td>40 F</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>11 Healthy</td>
<td>24 M</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>12 Healthy</td>
<td>23 M</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>13 Healthy</td>
<td>49 F</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>14 Healthy</td>
<td>41 F</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>15 Healthy</td>
<td>41 M</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>16 Healthy</td>
<td>45 M</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>17 Healthy</td>
<td>45 F</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>197</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>18 Healthy</td>
<td>52 F</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) 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.

**TABLE 1. Subject age, sex, pap smear, biopsy, and ELISPOT results**
isolated and analyzed separately using blood samples from assay to measure the numbers of HPV16-specific T cells. Syn/H9253/H9253 both are capable of secreting IFN-

generator-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 and addition of exogenous rhIL-2. 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.

ACKNOWLEDGMENTS

This work was supported by the University of Arkansas for Medical Sciences College of Medicine Individual Pilot Study Fund and the Arkansas Biosciences Institute, the major component of the Tobacco Settlement Proceeds Act of 2000.

We thank the subjects for participating in our study and Scott Roaf and Zhixiang Qu for their technical expertise.

REFERENCES


