Development, characterization and distribution of adoptively transferred peripheral blood lymphocytes primed by human papillomavirus 18 E7 - Pulsed autologous dendritic cells in a patient with metastatic adenocarcinoma of the uterine cervix

A. D. Santin[1,5], *M.D.*; **P. L. Hermonat**, *Ph.D.*; **A. Ravaggi**[5], *Ph.D.*; **S. Bellone**[5], *Ph.D.*; **C. Cowan**[2], *M.D.*; **S. Korourian**[3], *M.D.*; **S. Pecorelli**[5], *M.D., Ph.D.*; **M. J. Cannon**[4], *Ph.D.*; **G. P. Parham**[1], *M.D.*

[1]*Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, University of Arkansas, Little Rock, AR;*
[2]*Department of Nuclear Medicine, University of Arkansas, Little Rock, AR;*
[3]*Department of Pathology, University of Arkansas, Little Rock, AR;*
[4]*Department of Microbiology and Immunology, University of Arkansas, Little Rock, AR (USA);*
[5]*Division of Gynecologic Oncology, University of Brescia, Brescia (Italy)*

Summary

We describe a 27-year-old woman with systemic chemoresistant and radioresistant metastatic disease secondary to a recurrence of human papillomavirus (HPV) 18 infected cervical adenocarcinoma of the uterine cervix who received adoptive transfer of peripheral blood T cells stimulated with HPV 18 E7-pulsed autologous dendritic cells (DC). Extensive *in vitro* characterization of the DC-activated T cells derived from peripheral blood mononuclear cells (PBMC) included phenotypic analysis, cytotoxicity and intracellular cytokine production. High cytotoxicity activity was observed by CD8+T cells against autologous tumor cells, but not against NK-sensitive K562 cells, autologous Con-A lymphoblasts, or autologous Epstein-Barr virus-transformed lymphoblastoid cells. Blocking studies demonstrated that lytic activity was significantly inhibited by pretreatment of tumor targets with MAb specific for HLA class I as well as that of effector cells with anti-CD8, anti-LFA-1, but not anti CD3 MAb. Two-color flow cytometric analysis of the cytotoxic T cells revealed that a significant proportion of CD8+ cells was also CD56+. These double positive CTLs were thymically derived, as shown by expression of heterodimeric CD8 molecules (α/β CD8) and were endowed with high cytotoxic activity against tumor cells. Analysis of intracellular cytokine expression showed that the striking majority of E7-pulsed DC activated CD8+ T cells strongly expressed IFN-γ, TNF-α and IL-2 but not IL-4. The patient received two infusions of cytotoxic tumor-specific T cells at 2 week intervals, and *in vivo* distribution of the T cells was followed by ^{111}In oxine labeling and serial gamma camera imaging. Persistent accumulation of radioactivity in the lungs, which harbored extensive metastatic disease, was detected up to 120 hrs after the infusion. Taken together, these results illustrate the potential of E7-specific and tumor-specific CTL-based immunotherapy for the treatment of patients with invasive cervical cancer.

The abbreviations used are: HPV, Human Papillomavirus; MHC, Major Histocompatibility Complex; CTL, Cytotoxic T Lymphocyte; LCL, Lymphoblastoid B-cell line; DC; dendritic cells.

Key words: Cervical cancer; HPV; CTLs; Dentritic cells; Adoptive immunotherapy.

Introduction

Human papillomavirus (HPV) infection represents the primary risk factor for the development of cervical cancer [1]. Recent accumulating evidence suggest that the majority of cervical squamous cell carcinomas (SCC) and a large proportion of adenocarcinomas share a common pathogenesis involving oncogenic HPV types 16 and 18. Over 90% of SCCs have been shown to contain HPV DNA, while the range of HPV positivity in adenocarcinomas has been reported to be between 18% and 75% (for review see 2). E6 and E7 transforming oncoproteins of these two viruses are detected in a large majority of HPV-positive cancer biopsies and almost all HPV-containing cell lines, and play a crucial role in both transformation and maintenance of the malignant phenotype (for review see 2). Therefore, E6 and E7 viral proteins could be ideal candidates as potential tumor-specific targets for cervical cancer immunotherapy.

Dendritic cells (DC) are the most effective professional antigen presenting cells (pAPC) at activating naive T cells [3, 4] and recently the combination of GM-CSF and IL-4 has been shown to generate large numbers of DCs for stimulation of autologous human T cell responses [5]. In this study, we have used autologous DCs pulsed with full-length HPV 18 E7 oncoprotein in an attempt to generate a cytotoxic HPV-specific T cell response that also recognizes and kills tumor cells from a patient with adenocarcinoma of the cervix. In addition, after extensive *in vitro* characterization of the E7-specific CD8+ cytotoxic T lymphocytes (CTL), we performed adoptive transfusions to the patient. At that time, the patient had large metastatic tumor masses in multiple organs secondary to a systemic cervical adenocarcinoma recurrence that had

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failed all conventional treatment modalities and had brought the patient to an advanced cachectic state.

Patients and Methods

A 27-year-old woman presented in March 1998 with multiple systemic metastases secondary to a recurrence of HPV 18-positive cervical adenocarcinoma. In 1996 she was diagnosed with stage Ib2, grade 3 adenocarcinoma of the uterine cervix. At that time, she underwent a radical hysterectomy with bilateral salpingo-oophorectomy and pelvic lymphadenectomy. The final pathology report demonstrated an 8 mm invasion along the cervix with possible lymph space involvement. All the excised pelvic lymph nodes were negative for metastatic disease. No additional therapy was recommended at that time. In June 1997, she was found to have multiple metastatic lesions in both lungs consistent with recurrent adenocarcinoma of the uterine cervix. This diagnosis was confirmed with a computerized tomography (CT) guided needle biopsy. She subsequently underwent multiple courses of chemotherapy (6 courses of Cisplatinum, Ifosfamide and Bleomycin, 1 round of single Cisplatinum infusion, and 1 round of 5-Fluorouracil, Cisplatinum and Adriamycin). In addition, she received brain radiation, and left and right pelvic radiation, for cerebral and bone marrow pelvic metastases, respectively. After an initial response to chemotherapy, disease progressed during treatment. At the time of the beginning of the adoptive treatment with E7-pulsed DC-activated T cells, the patient had large and multiple systemic metastatic lesions in the brain, kidney, bone marrow, lungs and lumbo-aortic lymph nodes and was in an advanced cachectic state.

Tumor cells

Fresh autologous tumor cells were obtained from multiple punch biopsies obtained at the time of surgical treatment. Fresh tumor cells were prepared as described [6], and cultured in serum-free keratinocyte medium (KSFM-GibcoBRL), supplemented with 5 ng/ml epidermal growth factor and 35 to 50 µg/ml bovine pituitary extract (Gibco Life Technologies, Grand Island, N.Y.) at 37°C, 5% CO_2. PCR analysis on fresh tissue biopsies and tumor cell cultures revealed that tumor cells harbored the HPV 18 genotype. All the experiments testing in vitro cytotoxicity before reinfusion (see below) were performed with fresh tumor cultures which contained >99% pure tumor cell populations.

Dendritic cell cultures and generation of HPV E7-specific T cells

Peripheral blood mononuclear cells (PBMC) were obtained by leukopheresis two months after the radical hysterectomy in 1996. The derivation of dendritic cells from the patient's PBMC, and their subsequent use for generation of HPV E7-specific T cells, was carried out essentially as described [6]. Functional assessment of E7-specific T cells, including phenotypicanalysis, flow cytometric analysis of intracellular cytokine expression, and cytotoxicity assays against autologous tumor cells, was also conducted as previously described [6]. Expression of CD56 and the CD8 β chain was assessed by flow cytometry, using the Leu-19 (Beckton-Dickinson, San Jose, CA) and MCA 1722 (Serotec, Oxford, UK) MAbs, respectively. Demonstration of HLA class I restriction of tumor specific CD8+ T cell responses was achieved in standard cytotoxicity assay [6] in the presence of a blocking MAb specific for a non-polymorphic HLA class I determinant (W6/32) (50 µg/ml). In addition, to determine the structures on the effector cells involved in lysis, monoclonal antibodies against CD8 (5 µg/ml) (Ortho Pharmaceutical Corp, Raritan, NJ), CD3 (10 µg/ml), CD11a/LFA-1 (10 µg/ml) and its isotype control IgG1K MAb isotype standard anti TNP (10 µg/ml) (Pharmingen, San Diego, CA) were used to block cytotoxicity.

^{111}Indium-oxine labeling procedure

A fraction of the activated lymphocytes (from $4x10^7$ to $80x10^7$ cells) was used for labeling. Briefly, cells were radiolabeled by incubation with 800 µCi of ^{111}In-oxine (Amersham, Arlington Heights, IL) in 5 ml of PBS for 20 min at room temperature. The labeled cells were washed twice in autologous plasma, and before reinfusion were resuspended in 5 ml of normal saline plus 5% autologous plasma. Analog and digital gamma camera images were obtained at 4, 24, 48, 72 and 120 hours after T-cell injection using a ADAC Vertex (ADAC, Milpitas, CA) and Siemens Diacam (Siemens, Chicago, IL) equipped with a medium-energy-parallel-hole collimator for static imaging and a diverging collimator for whole-body scanning. Twenty percent windows were centered over the 173 and 247 KeV photopeaks of ^{111}Indium 10-minute regional scintiphotos of the anterior, posterior, right and left chest, abdomen and pelvis were recorded on film and computer.

Regions of interests were manually drawn around computer-digitized images of both lungs and liver from the anterior regional scintiphotos. Lung to liver ratios of radioactive counts were normalized for region size and decay-corrected for comparison of tracer uptake at serial time points.

Safety testing

Lymphocyte cultures were routinely checked before each infusion to exclude bacterial and fungal contamination. Standard Gram staining and culture analysis were performed. In additon, lymphocyte products were tested for mycoplasma contamination as well as endotoxin levels. No culture was observed to be contamined with mycoplasma, and the level of endotoxin did not exceed 1 endotoxin U/ml of culture medium.

Results

In vitro assessment

Extensive characterization of the peripheral blood lymphocytes primed by HPV 18 E7-pulsed DC was performed in vitro before the in vivo therapy. HLA-typing of the patient was found to be positive for HLA A1, A2, B7, B41, CW2, CW7. In addition, phenotypic expression of surface antigens on fresh cervical tumor cells was measured by flow cytometry. In this regard, autologous tumor cells expressed HLA class I but were negative for HLA-DR (data not shown). T cells were stimulated from PBMC collected by leukopheresis before the clinical appearance of recurrence (two months after surgical debulking). Almost all of the cultured lymphocytes expressed CD3 antigen on the surface (85% to 95%). Full-length E7 pulsed-DC-activated lymphocytes consisted of subpopulations with predominance of CD4+ T cells (range from 55 to 75%) over CD8+ T cells (range from 28 to 47%). Further activation with OKT3 and feeder cells produced an average 100-fold increase in cell number after 2 weeks and increased the number of TcR γ/δ positive cells from 2% to 7%. CD56+ cells ranged

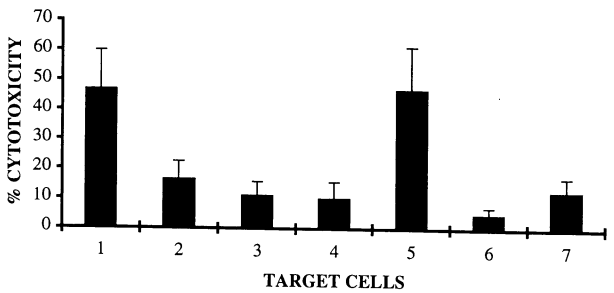


Figure 1. — Tumor specific CD8+ CTL responses induced by HPV18 E7-pulsed DCs measured in a 6 h 51Cr-release assay. Percentage lysis (± standard deviation) at a 20:1 effector/target cell ratio is shown. 1, Autologous tumor; 2, Autologous tumor + W6/32 anti-Class I MAb; 3, Autologous tumor + anti-CD8 (OKT-8) MAb; 4, Autologous tumor + anti-CD11a (LFA-1) MAb; 5, Autologous tumor + anti-CD3 MAb; 6, Autologous LCL; 7, K562.

from 10 to 27%. In vitro cytotoxicity assays were conducted using pure CD8+ populations (i.e., more than 98% CD8+). As shown in Figure 1, significant cytotoxicity against autologous tumor-cell targets was demonstrated in several separate assays. In this regard, cytotoxicity ranged from 25 to 65% at 20 effectors per target while the level of cytotoxicity against the NK-sensitive cell line K562 was always at a lower level (Fig. 1). The absence of cytotoxicity against autologous Con-A lymphoblasts (data not shown) or autologous EBV-transformed LCL (Fig. 1) showed that, although these cells were highly cytotoxic against autologous tumor cells, they failed to kill normal cells or autologous cells infected with EBV. Blocking studies demonstrated in all cases that the tumor-specific lytic activity against autologous tumor targets was significantly inhibited by pretreatment of tumor targets with MAb specific for HLA class I (W6/32) (range of inhibition: from 30 to 65%) (Fig. 1) but not with pretreatment of target cells with isotype controls (data not shown). These results are broadly in agreement with our previously reported characterization of E7-specific CD8+ T cell responses from cervical cancer patients [6]. We also found that MAbs specific for anti-CD11a (LFA-1) and anti-CD8 (OKT-8) but not anti-CD3 MAb were also able to block tumor lysis to a significant extent, the range of inhibition being from 50 to 76% and from 59 to 67%, respectively. These findings suggest that the CD8 co-receptor molecule and CD11a-CD54 adhesion pathway are critical for effective CD8+ T cell mediated lysis of cervical tumor target cells (Fig. 1). Interestingly, when the expression of CD56 on T lymphocytes was future analyzed by two color immunofluorescence we found that different percentages of CD8+ T lymphocytes (range from 15 to 53%) co-expressed the CD56 surface antigen during culture (Fig. 2). The percentage of CD56 expression has previously been reported to be strongly correlated with a high cytotoxic activity [6]. Recently, a novel population of T cells co-expressing the CD8+ and CD56+ markers and endowed with a potent antitumor activity have been described in mice as well as humans (10-12). The origin of these cells expressing both the T cell markers CD3/CD8 and the NK cell marker CD56, is still uncertain, but recent reports have suggested that these T cells could be extrathymically differentiated [10-12]. In this regard, homodimeric CD8α/α expression has been previously shown to be inducible in multiple cell types by activation events in the periphery, whereas T cells of thymic origin express the CD8α/β heterodimer [13, 14]. To discern between the thymic or extrathymic origin of these highly cytotoxic CD8+/CD56+ T cell populations, we stained the pure populations of CD8α/CD56+ T cells with Ab against the β chain of the CD8 molecule [13, 14]. As can be seen in Figure 2 we found that all of the CD8α+/CD56+ CTL expressed the CD8α/β heterodimer, confirming the thymic origin of these highly cytotoxic T cells.

Finally, we took advantage of a recently developed flow cytometric technique for detecting intracellular cytokine expression at the single cell level in the tumor specific CD8+ T cell populations. Two-color flow cytometric analysis of intracellular IFN-γ, TNFα, IL-2 and IL-4 expression of the CTLs demonstrated a striking dominance of Type 1 cytokine phenotype (Fig. 3). These data, therefore show a strong correlation between Type 1 cytokine production by HPV/tumor specific CD8+ T cells and high cytotoxic activity against tumor cells.

In vivo localization

Before the study, the patient had failed all conventional treatment modalities, as well as chemotherapy and radiotherapy salvage treatments, and late stage metastatic disease had brought her to an advanced cachectic state. At the time of adoptive T cell therapy, large tumor metastases were detectable by chest X-ray and CT scan in the lungs (Fig. 4 A and B), as well as the kidneys, brain, pelvic bone marrow and lumbo-aortic lymph nodes (data not shown). Because the tumor specific DC-activated lymphocytes consisted of subpopulations of CD4+ as well as CD8+ T cells, and the patient was at high risk for respiratory dysfunction due to the advanced metastatic tumor spread in the lungs, no recombinant IL-2 was administered with the T cells. Both injections were infused intravenously through a central line. The total numbers of lymphocytes delivered were $2x10^8$ and $5.8x10^8$, respectively, while the number of ^{111}In-radiolabeled lymphocytes ranged from $4x10^7$ to $8x10^7$ cells, respectively. Cell viability of the DC-activated lymphocytes after radiolabelling was 98%±1% (range, 96-99%). Four hours after intravenous bolus infusion, gamma camera imaging showed exclusive distribution of radioactivity in the lungs, liver and spleen (Fig. 5). The greatest amount of uptake was measured in the liver and spleen at all time points tested thereafter. Serial images obtained at up to 120 hrs revealed that although pulmonary uptake cleared significantly during the first 24 hrs, persistent radioactivity remained detectable in the lungs throughout the study period (Fig. 5). When the lungs to liver ratios of radioactive counts were normalized for region size and decay corrected, approximately 60% of

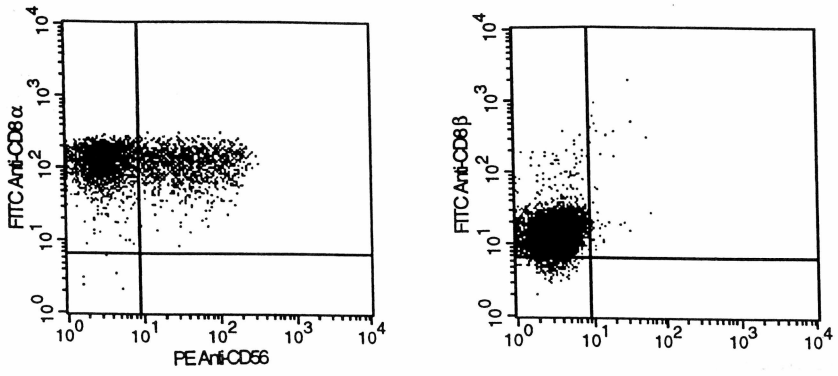


Figure 2. — Flow cytometric analysis of CD56 expression by tumor-specific CD8+ T cells (left panel) and CD8β chain (right panel). A representative experiment is shown.

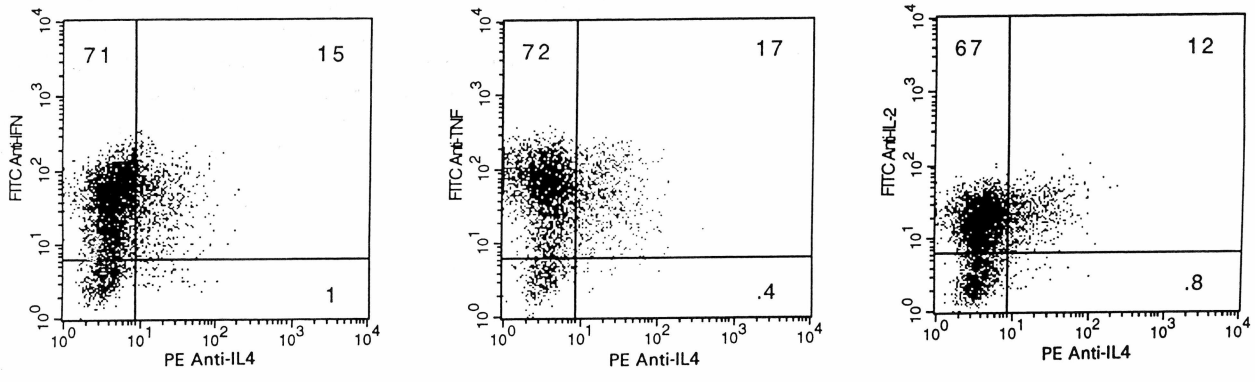


Figure 3. — Two-color flow cytometric analysis of intracellular IFN-y vs IL-4 (left panel), TNF-a vs IL-4 (middle panel), IL-2 vs IL-4 (right panel) expression by tumor specific CD8+ T cells. T cells were activated with PMA and ionomycin 14 days after the last antigen stimulation.

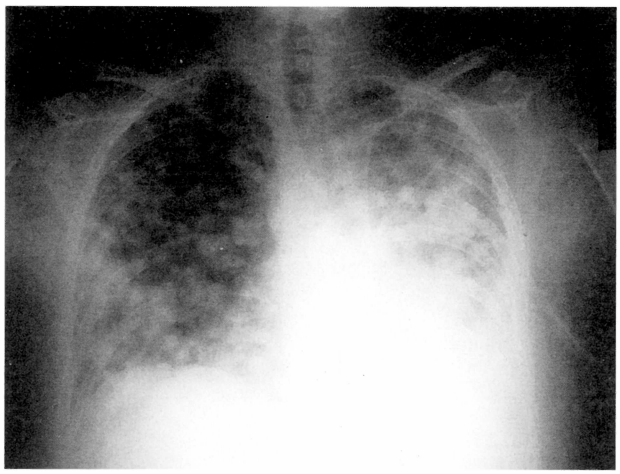
residual activity was found in the lungs at 24 to 120 hrs when compared to 4 hrs (Fig. 5). These data, therefore suggested a specific localization and persistent accumulation of the E7-pulsd DC activated CTL in the lungs harboring the extensive metastatic disease (Figs. 4 and 5). Distribution of ^{111}In DC-activated lymphocytes in bone marrow and lumbo-aortic lymph nodes was also observed at 24-hr images, and showed a steady rise over the first 72 hrs (data not shown). Infusions of DC-activated lymphocytes were not associated with any significant side-effects.

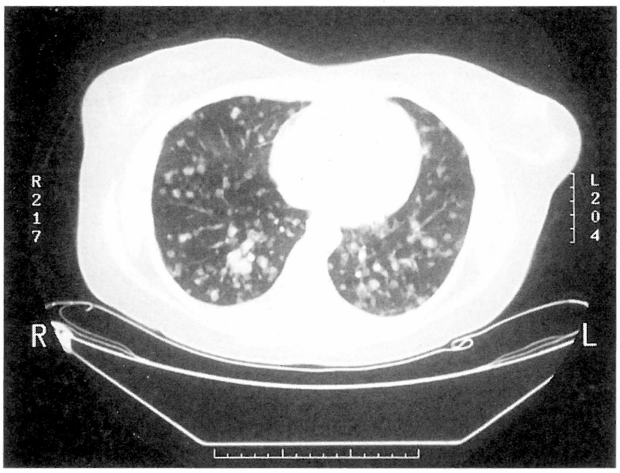
Discussion

Several lines of evidence derived from epidemiological and experimental studies suggest that cell-mediated immune responses are important in controlling both HPV infections and HPV-associated neoplasms [for review see 1 and 2]. Despite these observations, to date, only a few reports of HPV-specific CTL responses in humans have been documented. This has led to the suggestion that HPV has coadapted to the human host by evading the immune system. However, HPV-infected epithelial cells could fail to generate CTL responses effectively, not for the lack of specific CTL precursors, but because the virus tightly controls the expression of antigenic proteins in the replicating cells that are subjected to T cell surveillance, (e.g. basal epithelia) [15]. Therefore, because very little transcriptional activity and low numbers of viral genomes are presented in infected basal cells [16, 17], only a minimal amount of viral antigens might be available for an efficient presentation by epidermal professional DC. In agreement with this hypothesis, antigen load has been previously shown to be a decisive factor in the generation of HPV specific T lymphocytes responses [18]. In addition, immunological ignorance of HPV oncoproteins with respect to a CTL response has recently been reported in transgenic mice expressing E6 and E7 oncoproteins in keratinizing epithelia [19]. Nevertheless such mice were immunized by E7-based peptide vaccination and were protected against a subcutaneous challenge with E7-positive tumor cells [19].

In this paper we report the distribution of E7-pulsed DC-activated lymphocytes in a patient with a systemic recurrence of a highly aggressive HPV18 infected adenocarcinoma of the uterine cervix. Functional characterization of the DC-activated E7-specific T cells showed that the CD8+CTL response was capable of killing the patient's autologous tumor cells in a highly specific and

A





B

Figure 4. — Representative X-ray **(A)** and CT scan **(B)** images of the patient's chest 1; Scans obtained 1 weeks before the first treatment. Note the extensive metastatic lesions in both lungs.

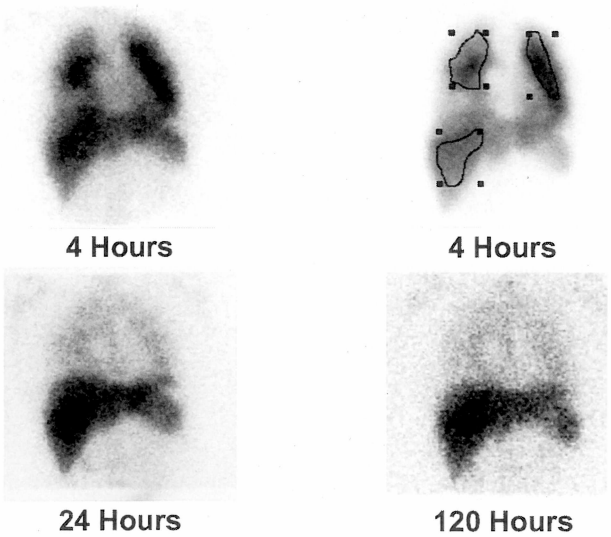


Figure 5. — Representative anterior scintiphotos at 4, 24 and 120 hrs of the chest. Regions of interest used to calculate lungs to liver radioactive signal ratios at different time points are also shown in the scintiphoto at 4 hrs. Note that qualitatively the heart is a cold shadow after 24 hrs, indicating more than normal residual activity in the lungs. Quantitatively, lung to liver ratios of radioactivity counts, normalized for region size and decay corrected, show approximately 60% residual activity in the lungs at 24, 48 (not shown) and 120 hrs compared to 4 hrs.

HLA class I-restricted manner. In addition, MAbs specific for anti-CD11a (LFA-1) and anti-CD8 (OKT-8) were also able to significantly block tumor lysis. These data, therefore, support, in addition to HLA Class I restriction elements, an important role played by the CD8 molecule, which is also known to be involved in T cell-target cell interactions [20], and the LFA-1 molecule, which can act as accessory receptor able to increase TCR-dependent target cell binding [21], in lysis of HPV-infected autologous tumor cells.

Interestingly, a significant proportion of the CD8+ T cells also expressed the natural killer cell marker CD56. This observation is in agreement with our previous findings (6) as well as those of others [22], showing a significant correlation between high CD56 expression on CD8+ CTL and cytotoxic activity against cervical cancer. Recently, a novel population of CD8+/CD56+ cells derived from T cells with potent antitumor activity have been described in humans [10-12]. However, the origin of these cells expressing both the T cell markers CD3/CD8 and the NK cell marker CD56 is still uncertain, but recent reports have suggested that these T cells could be extrathymically differentiated [10-12]. To investigate this hypothesis we stained the populations of CD8α/CD56+ T cells with a MAb against the β chain of the CD8 molecule. In this regard, extrathymically differentiated T cells have been reported to preferentially express the CD8α/α homodimer, rather than the α/β heterodimer that is restricted to populations generated in the thymus [13, 14]. We found that in our culture conditions the CD56+CTL were heterodimeric for the CD8 molecules (CD8α/β), therefore confirming the thymic origin of these highly cytotoxic and HLA-restricted T cells. We would suggest, therefore, that CD56 expression by CD8+CTL may be an activation antigen associated with high cytotoxic function, rather than a lineage-specific marker.

Finally, using two-color flow cytometric analysis of intracellular cytokine expression by CD8+ T cells, we found that DC-stimulated tumor-specific T cells showed a striking dominance of Type 1 cytokine expressors. These findings, therefore, indicated a strong correlation between high cytotoxic activity against autologous tumor cells and IFN-γ, TNF-α and IL-2 secretion by E-7 specific CD8+ CTLs.

The homing and migration of normal lymphocytes as well as in vitro activated tumor infiltrating lymphocytes (TIL) has been studied extensively [23, 24]. In contrast, the migration potential of tumor specific DC-activated lymphocytes after adoptive transfer to cervical cancer patients has not been studied. ^{111}In-labeled-PBL infused

in normal human volunteers as well as ^{111}In-labeled-TIL infused in patients with melanoma have been shown to result in initial uptake in the lungs, then clearing by 24 hrs, followed by stable uptake in the liver and spleen [23, 24]. In agreement with these observations, after i.v. infusion of ^{111}In-labeled DC-activated lymphocytes, significant accumulation of radioactivity in the lungs as well as spleen and liver was readily detectable by gamma camera images at 4 hrs. Furthermore, persistent accumulation of activity in the lungs was detectable throughout the study period (e.g. 120 hrs), and quantification of lungs to liver ratios of radioactive counts, normalized for region size and decay corrected, showed 60% of residual activity after 24 hrs up to 120 hrs. These data, therefore, suggested a specific localization and persistent accumulation of E7-pulsed DC activated T cells around the extensive lung metastases. Because of the advanced cachectic state of the patient and the short time available for the expansion of E7-specific DC activated lymphocytes to be used for adoptive transfer, only a relatively low number of E7-pulsed DC activated lymphocytes were available for reinfusion. This may explain the lack of a significant clinical effect against the large amount of metastatic tumor harbored by the patient. The infusions of DC-activated lymphocytes were not associated with any significant side-effects, confirming the safety of this approach.

DC are the most powerful APC known in humans, and their manipulation has already shown significant therapeutic effects in several established human tumor models [25, 26]. The ex-vivo manipulations required to produce tumor antigen-pulsed autologous DC-based vaccines are safer, less expensive and less cumbersome than those required for the generation of genetically engineered autologous tumor cell vaccines. In addition, the presence of a well defined tumor antigen (e.g. HPV E7) that is constantly expressed by cervical tumor cells as a defined CTL target antigen, as well as the readily available supply of recombinant E7 oncoprotein from the high risk HPV genotypes renders this approach feasible for large scale applications. Taken together, the findings presented in this paper indicate that it is possible to elicit HPV-specific CTL with E7-pulsed DC against autologous cervical cancer tumor cells in vitro, and these activated lymphocytes may have the potential to traffic to the tumor site in vivo. Further studies in patients at an earlier stage of disease or with a lesser tumor burden will be necessary to evaluate whether E7-pulsed DC-based vaccination or the adoptive transfer of in vitro generated E7-specific CTL hold the potential for therapeutic benefit with HPV positive cervical carcinoma.

Acknowledgments

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Address reprint requests to:
ALESSANDRO D. SANTIN, M.D., UAMS
Medical Center, Division of Gynecologic Oncology
University of Arkansas
4301 W Markham
Little Rock, AR, 72205-7199 USA