RESEARCH ARTICLE

Development and Clinical Evaluation of Dendritic Cell Vaccines for HPV Related Cervical Cancer - a Feasibility Study

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Abstract

Human papillomavirus infection (HPV) and HPV related immune perturbation play important roles in the development of cervical cancer. Since mature dendritic cells (DCs) are potent antigen-presenting cells (APC), they could be primed by HPV antigens against cervical cancers. In this study we were able to generate, maintain and characterize, both phenotypically and functionally, patient specific dendritic cells in vitro. A randomized Phase I trial with three arms - saline control (arm I), unprimed mature DC (arm II) and autologous tumor lysate primed mature DC (arm III) and fourteen patients was conducted. According to WHO criteria, grade 0 or grade one toxicity was observed in three patients. One patient who received tumor lysate primed dendritic cells and later cis-platin chemotherapy showed a complete clinical response of her large metastatic disease and remained disease free for more than 72 months. Our findings indicate that DC vaccines hold promise as adjuvant sfor cervical cancer treatment and further studies to improve their efficacy need to be conducted.

Keywords: Dendritic cells - immunotherapy - cancer vaccines - cervical cancer - HPV

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Introduction

Cancer of the uterine cervix is the most common gynaecological cancer (Saumya Pandey et al., 2012) and the leading cause of mortality from cancer among women worldwide, with more than 80% of the cases occurring in developing countries like India (Ferlay et al., 2010). A causal link has been established between infection with human papillomavirus (HPV) and cervical cancer (Das et al., 2012). A majority of women infected with HPV clear the infection, but in some of them it persists and progresses to cancer. Due to lack of screening, awareness and limited access to treatment in developing countries, a large number of cases of cervical cancer present at an advanced stage.

Cervical cancer patients are now known to have a number of immune system defects ranging from down-regulation of MHC (Major Histo compatibility) class I molecules and abnormal upregulation of MHC class II molecules (Cromme et al., 1994) to transcriptional downregulation of mRNA of anti-tumorigenic cytokines like IFN-γ (Tartour et al., 1998). Hence there is a lack of relevant antitumor response which is essentially mediated by specialised antigen presenting cells called dendritic cells (DC). Some tumors may also secrete inhibitory cytokines like IL-6, which prevent maturation of local dendritic cells (DC) (Srivani and Nagarajan, 2003).

Various strategies have been investigated for stimulating the immune system in cancer patients with advanced disease. Therapeutic vaccination strategies for solid cancers based on tumor associated peptides have failed to elicit significant clinical responses in several trials (Claesson, 2009). In the case of cervical cancer, it has been suggested that the presence of HPV viral peptides could be used as a basis for a 'vaccine' in women in whom all other treatment has failed. However the presence of the viral peptides does not make a cancer immunogenic, as found in studies that aimed at studying CD4+ T cell mediated immunity against HPV E2 and E6 epitopes (de Jong et al., 2004).

Dendritic cells (DCs) are the most potent antigen-presenting cells (Banchereau and Steinman, 1998), and the viral peptides present in cervical cancer could be used specifically to prime DCs. But due to the presence of more than 100 different HPV types and lack of knowledge about cross protection, using a HPV specific peptide may not be effective in clearing an existing tumor. Hence, whole-cell antigens might be more effective, as the virus does not affect DCs, and cross-priming is an efficient way of activating DCs. Furthermore, DCs could stimulate a CTL response (Xiang Mai Wu et al., 2014).The objective of the study reported here was to determine the feasibility of generating DC *in vitro*, evaluating the best strategy for antigen pulsing and then conduct a phase-one trial in advanced, recurrent cervical cancer patients who had

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failed conventional therapy. The tumors needed to be HPV positive and toxicity was the primary endpoint. Secondary endpoint of the study was monitoring immunological responses in vaccinated patients.

Materials and Methods

Evaluation of antigenic sources for pulsing

Dendritic cells were generated *in vitro* for evaluating different antigen priming strategies. After obtaining institutional ethical committee clearance and an informed consent 30-ml blood and punch biopsy samples were collected from cervical cancer patients before they underwent treatment. PBMC separated using a Ficoll Paque plus density gradient (GE Healthcare, Amersham, UK) were washed and plated with serum-free AIM-V medium (Gibco BRL, Grand Island, New York, USA) for monocyte enrichment at 37°C in 5% CO_2 for 2 h. After 2h the non-adherent fraction was removed and Fresh medium containing IL-4 and GM-CSF (Sigma Aldrich, St Louis, Missouri, USA) was added at 100 ng/ml concentration each, and cultured for 7 days at 37°C in 5% CO_2. Medium containing cytokines was replenished on the third and fifth day. On the seventh day, immature DC floating were transferred in fresh AIM-V medium to a six-well plate for antigen loading and maturation.

Antigen loading was evaluated using three strategies- tumor lysate, cervical cancer cell line lysate and tumor RNA. Cervical tissue samples were obtained by punch biopsy and minced with a surgical scalpel blade and then digested with a cocktail of enzymes- collagenase (0.07%) and hyaluronidase (0.1%) in HBSS (both from Sigma) at 37°C/10% CO_2. Cell lysate was prepared by alternating cycles of freezing with liquid nitrogen and thawing in a water bath at 56°C. A trypan blue exclusion test was done to confirm cell death. Tumor lysate was added to wells containing immature DC at ratio of 1:0.5, 1:1 and 1:3 (DC: tumor cell ratio) in order to determine the optimal priming ratio.

For priming with cell line cocktail lysate, the cervical cancer cell lines HeLa, SiHa and C33A were chosen. For RNA pulsing, total RNA was isolated from a punch biopsy obtained in RNAlater® (Ambion, Austin, Texas, USA). For RNA extraction, Trizol reagent (Invitrogen, San Diego, California, USA) was used according to manufacturer's instructions. The final pellet was dissolved in RNA Secure® (Ambion) and added to the immature DC at a concentration of 5 μg /ml.

After 4 h of exposure to antigen, IL-1β and TNF-α (100ng/ml-both from Invitrogen, USA) were added the cells were incubated for 3 days at 37°C under 5% CO_2. A fraction of the cells (1x105) were phenotyped by flow cytometry and the remaining cells were assessed for their functional activity in a mixed lymphocyte reaction (MLR).

Flow cytometry

Immature and mature DCs were labelled with the primary antibodies HLA-DP, DQ, DR (clone CR3/43) dilution 1:75, CD86 (clone BU63) dilution 1:25 and FITC-CD14 (clone TUK4) 4 μl (all from Dako, Glostrup, Denmark) for 30 min. After incubation, the cells were washed with 2% BSA-PBS, and FITC anti-mouse rabbit secondary antibodies (also from Dako) were added to the control tube and unstained primary antibody containing tubes alone, washed and finally fixed in 0.5% paraformaldehyde. Acquisition and analysis were done on a BD FACS Calibur flow cytometer using the Cell Quest Pro software.

Mixed lymphocyte proliferation assay

Graded numbers of (0-10 000) irradiated (24 Gy) mature dendritic cells were added to 2×10^4 cells of T cells from allogeneic donors in a 96-well tissue culture plate in triplicates. Stimulation of responding T cells was determined after 5 days of co-culture. Cell Titer 96 aqueous one-solution proliferation assay (Promega, Madison, Wisconsin, USA) was done following the manufacturer's instructions. Readings were taken at 492 nm to determine the percentage cell proliferation, with wells containing no DC as baseline. Positive control cells were stimulated with phytohemagglutinin (Gibco) at a concentration of 5μg/μl on the second day. Unstimulated cells were used for calculating the percentage proliferation.

Patient recruitment for the Phase I trial

The clinical trial was done from 2004-2008, at a time when the registration for the clinical trials in India was not mandatory. It became mandatory from June 2009 and by then the study had been completed. Fourteen patients were enrolled in the study after an informed consent had been obtained, and the trial design was approved by the Institutional Ethical Committee. The schema for recruitment in the clinical trial is given in Figure 1 The eligibility criteria were a histologically confirmed diagnosis of cervical cancer with HPV presence detected by polymerase chain reaction (PCR) using MY09 and MY11 consensus primers and local or distant recurrent malignancy after initial radical treatment, a Karnofsky score ≥70, normal baseline haematological parameters (within 1 week of first vaccination) - hemoglobin>9.9 g/dl total granulocyte count>1000/μl, platelet count>60

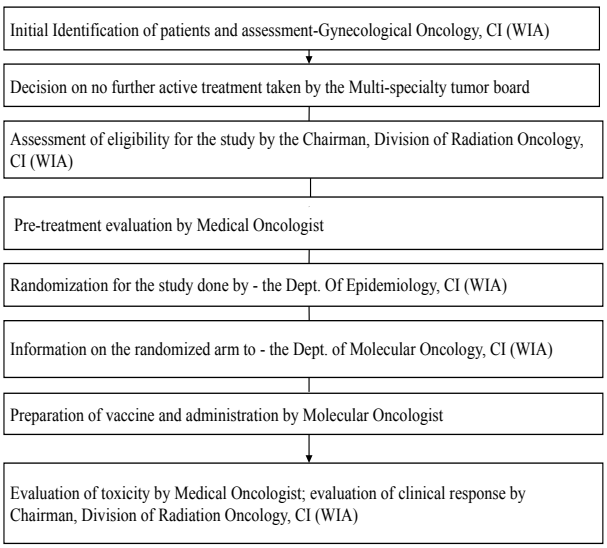

Figure 1. Schema for Recruitment of Patients for the Phase I Clinical Trial

000 /µl, blood urea nitrogen<30 mg/dl, creatinine<2 mg/dl, alkaline phosphatase and aspartate aminotransferase less than twice the upper limit of normal, prothrombin time and activated partial thromboplastin time ≤ 1.4 times control, unless therapeutically warranted.

Patients were excluded if they were positive for HIV or hepatitis B or C, were pregnant, had severe pulmonary or cardiac disease, brain or spinal cord metastasis, uncontrolled diabetes, hypertension (if not optimally controlled before recruitment), an acute infection requiring active treatment, or a history of an auto-immune disorder or prior history of other malignancies.

Patients were randomized to one of three arms- Arm I –placebo arm in which the patient received saline alone. Arm II patients received mature dendritic cell vaccines without tumor lysate exposure [unprimed DC's]. Arm III patients received tumor lysate pulsed dendritic cell vaccines [primed DC's]. The arm I was later dropped after recruitment of 9 patients, as the Dept. of Biotechnology (funding agency) review committee found a satisfactory difference between patients in the placebo arm and those receiving dendritic cells (with or without antigen exposure).

Dendritic cell vaccine generation and phenotyping by flow cytometry

From patients recruited for the randomized Phase I trial, 75ml of blood was drawn and processed as done for the *in vitro* studies. They were matured on day 7 with or without antigen pulsing for three days with anti-inflammatory cytokines. On day 10, the mature DC was phenotyped as above and tested for microbial sterility.

Vaccination and dosage

The DCs that were found to be sterile for more than 72 h microbiologically were frozen in a mixture containing 85% autologous serum (heat-inactivated), 10% DMSO and 5% glucose. On the day of vaccination, the cells washed three times with saline and given to each patient intra-dermally. Patients received three vaccinations, one every 14 days. A maximum of 1x106 cells were given at each vaccination.

Clinical response and toxicity assessment

Although a clinical response was not expected, patients were evaluated through clinical examination before and after each vaccination and during subsequent follow up. X-ray or ultrasound was done when considered necessary. Adverse events were recorded using WHO common toxicity criteria.

DTH skin test

Lysates prepared from approximately 104 and 105 tumor cells were given intra dermally to patients 7 days after the last dose of vaccine along with saline intra dermally as a control. The response was observed at 24, 48 and 72 h and was considered positive only if the reaction had a diameter of more than 5 mm in any one dimension.

Proliferation assay

A total of 50 000 lymphocytes were plated in triplicate and stimulated with a lysate fraction containing an equal number of cells in 96-well plates and incubated at 37°C in 5% CO_2 for 5 days. Cells stimulated with PHA were used as a positive control, and unstimulated lymphocytes were used as the baseline for calculating the percentage of proliferation. On day 5, MTS Cell Titer One- (Promega, USA) assay reagent was added to the wells as per the manufacturer's instructions. The plates were incubated for 4 h at 37°C, and the colour developed was read at 492 nm.

Interferon-gamma, CD3 analysis by flow cytometry

Lymphocytes cryopreserved from the patients before vaccination and after the third vaccination were rested overnight, pulsed with tumor lysate at a 1:1 ratio and incubated at 37°C /5% CO_2 for 6h. After an hour, Golgi stop solution (Pharmingen, San Diego CA) was added to arrest cytokine secretion. The cells were stained with anti-CD3-PE, fixed and permeabilized with Cytofix-Cytoperm solution (Pharmingen), and stained with anti IFN-γ-FITC antibody. Isotype-matched controls were run and analysis was done on a BD FACS Calibur machine using Cell Quest Pro software.

Immunohistochemistry

Paraffin embedded sections of 10µm thickness from biopsy samples taken before and after the third vaccination were stained with antibodies against CD8 (C8/144B clone M7103 at 1/25 dilution), CD45RO (OPD4 clone M0834 at 1/50 dilution), CD20 (L26 clone M0755 at 1/200dilution), CD56 (T199 clone M0852 at 1/10 dilution; (DAKO corp., Glostrup, Denmark).

Antinuclear antibody ELISA

Plasma collected after Ficoll Paque (GE Life sciences) was stored at -70°C and used for evaluating any autoimmunity post vaccination. An ANA Detect kit (ORG 600) was used and an ELISA was done according to the manufacturer's instructions.

Results

Phenotypic characterization of DC generated in vitro

Our in vitro study results showed that immature DCs generated from monocytes showed increased HLA-DP, DQ, DR (++) expression, slightly increased CD86 levels (+) but almost no CD14 (-) expression. Mature DCs when compared to immature ones showed significant increase in HLA, DP, DQ, DR (+++) and CD86 (++) levels (Figure 2). Addition of TNF-α and IL-1β increased levels of HLA class II expression in the cell periphery along with that of CD86 costimulatory molecules.

Antigen loading strategies may influence functional capacity of DC

Mixed lymphocyte reaction with allogeneic donor PBMCs revealed that the cervical cancer cell line cocktail lysate (prepared from HeLa, SiHa and C33a) primed DCs showed increased stimulatory capacity but not significantly higher than tumor lysate primed DC (paired two tailed t test, p value=0.6) or RNA primed DC (p value=0.9) at 10,000 DC concentration. We observed in

our experiments that mature DC, irrespective of antigen loading strategy, definitely had better ability to stimulate PBMC compared to immature DC (Figure 3). Although not strictly significant, the autologous tumor lysate primed DC (p value=0.08) but not the autologous RNA (p=0.7) primed DC were better stimulators of PBMC when compared to unprimed immature DC at 10,000 DC concentration. Proliferation responses were significantly higher (Figure 4) for lysates added in a tumor cell: DC ratio of 3:1 than 0.5:1, (paired two-tailed t test, p= 0.01). Hence this ratio was determined to be ideal for culturing and maturing DCs in vitro for use in the phase I clinical trial. As the Institutional human ethics committee did not permit the usage of cell line lysate, autologous tumor lysate priming was chosen as the antigen loading strategy.

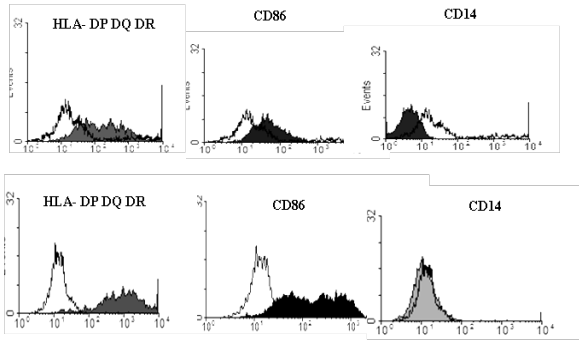

Figure 2. Dendritic Cell Phenotypic Characterization by Flow Cytometry- Figure. 2a. Immature DC phenotype-HLA DP, DQ, DR-++, CD86+, CD14+. **Figure 2b**. Mature DC phenotype-HLA DP, DQ, DR-+++, CD86+++, CD14-

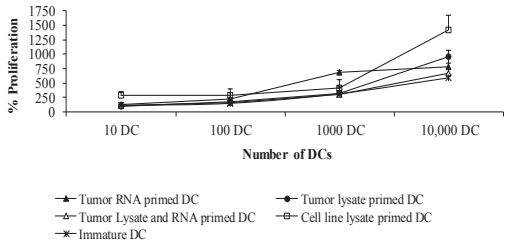

Figure 3. MLR Using DC Primed with Different Antigenic Sources. Mixed lymphocyte proliferation in the presence of graded numbers (10, 100, 1000, 10 000) of DC primed with different strategies and matured with inflammatory cytokines were used to stimulate 10 000 allogeneic donor PBMC. Wells containing only donor PBMC (10 000) were the baseline. Immature DC were also tested for their functional capacity. Data shown is summary of three independent experiments. Cell line lysate primed DC followed by tumor lysate primed DC induced greater proliferation than DC primed by other strategies

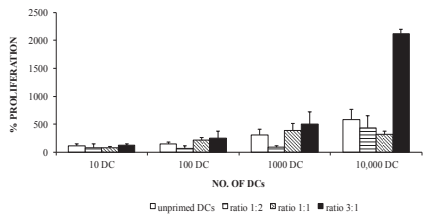

Figure 4. Dose Effect of Tumor Lysate for DC Priming in an MLR. Immature DCs were primed with tumor lysate at three different ratios and evaluated in a mixed lymphocyte reaction for their functional ability. At 10 000 DC concentration, maximum proliferation occurred for the 3:1(tumor cell: DC) ratio

Phase I trial patient characteristics

In all, nine patients were recruited for the three arm trial and five patients were recruited for the two arm trial after they were found to fit the eligibility criteria and provided an informed consent. Age range of the patients was 27-70 years (median-49 yrs.). All patients except one received standard treatment at Cancer Institute (WIA), Chennai, India. Patient 7 alone received the same at the Govt. General Hospital, Chennai, for cervical cancer. Other patient characteristics are summarized in Table 1. Two patients (3 and 11) had lung metastasis while Patient 10 had a left external iliac vessel metastasis,

DC vaccine yield, phenotype and dosage

Immature DC yield was found to depend on individual monocyte counts. But in arm III patients' tumor cell percentage influenced mature DC numbers. A maximum of 7.6×10^6 primed DCs could be generated in one arm III patient after pulsing with tumor lysate but this was not possible in all patients as tumor cell numbers and ratio of pulsing (3 tumor cells: 1 DC) limited the number of antigen primed DCs that could be generated. Tumor cell percentage was determined by a qualified cytologist from cytospin preparations after tissue digestion. This percentage was used to calculate the actual no. of tumor cells that could go to priming DCs in arm III and it varied from 20% to 86% (median-43%). All the patients who enrolled for the trial were previously treated and hence viable number of cells was also a limiting factor for generating mature lysate pulsed DC.

DC phenotype was consistent with our *in vitro* studies as indicated in Table 1. Following DC preparation and maturation with or without antigen loading, they were subject to microbiological tests of sterility including gram staining and cultures for a period of 72-96 h and were administered after being certified sterile by the Dept. of Microbiology, Cancer Institute (WIA). The dosage and arm of vaccination of each patient are also indicated in Table 1.

Clinical Outcome, toxicity and DTH response

The vaccine was well tolerated in all patients. WHO toxicity criteria were used for evaluation and accordingly patients had only grade 0 or grade 1toxicity. Nevertheless, any other symptoms experienced even if they were unrelated to vaccination, were also recorded and patients were managed accordingly. The DTH responses developed are summarized in Table 1. Table 2 summarizes the grade and type of toxicity seen. The tests were done in 11/14 patients. In patients 10, 13 and 14, DTH could not be done. Patient 10 developed mild icterus prior to DTH. Liver function tests revealed that she had mildly elevated levels of bilirubin and alkaline phosphatase (ALP). This patient had elevated ALP levels even at the time of recruitment but the levels were lower than twice the upper limit and was recruited as she fit the eligibility criteria. On further observation, her icterus subsided but her ALP level did not return to normal and hence DTH testing was not done. Patient 13 who had a pre-existing hydronephrosis saw

Table 1. Patient Characteristics, Arm of Vaccination, DC Phenotype and DTH Reaction to the Dendritic Cell Vaccine

Patient	Age	Stage#	Arm of vaccine	HLA-class II status	CD86 status	No. of DCs /dose	DTH with $1X10^4$ cells (mmxmm)	DTH with $1X10^5$ cells (mmxmm)	Patient status
1	70	III B	I	N.A	N.A	Saline only	NIL	NIL	Lost to follow up
2	50	II A	III	(+++)	(++)	$1.2X10^5$ cells/dose	NIL	48 h: 2X2.1/72 h: 2X2.5	Dead*
3	50	III B	III	(++)	(++)	$1X10^6$ cells in the I dose/0.6 million cells in the II and III vaccination	NIL	48 h: 9X4/ 72 h: 5X4	Alive and Disease free
4	50	II B	II	(+++)	(+++)	$0.6X10^6$ cells/dose	NIL	NIL	Dead*
5	50	II B	II	(+++)	(+++)	$1X10^6$ cells/dose	NIL	NIL	Dead*
6	35	II B	I	N.A	N.A	Saline only	NIL	NIL	Dead*
7	50	III B	II	(+++)	(++)	$1X10^6$ cells /dose	48 h: 9/72 h: 6	48 h: 14 /72 h: 8	Dead*
8	47	III B	I	N.A	N.A	Saline only	NIL	NIL	Lost to follow up
9	70	II B	III	(+++)	(++)	$1X10^6$ cells /dose	NIL	NIL	Lost to follow up
10	27	III B	II	(+++)	(+++)	$1X10^6$ cells /dose	was not done	was not done	Dead*
11	48	III B	II	(+++)	(+++)	$1X10^6$ cells /dose	48 h: 5X7/72 h: 4x6	48 h: 8X10/72 h: 5X8	Dead*
12	40	III B	III	(+++)	(++)	$1X10^6$ cells /dose	NIL	only mild erythema was noticed	Dead*
13	40	II B	II	(+++)	(+++)	$1X10^6$ cells /dose	was not done	was not done	Dead*
14	40	II B	III	(+++)	(++)	$1X10^6$ cells /dose - received one dose only	was not done	was not done	Dead*

*Due to disease progression; (+++) - >75% expression, (++) - >50-75% expression; #Original stage at presentation. Patient 5 and 10 alone had adenosquamous carcinoma while the others had squamous cell carcinoma

Table 2. Toxicity Observed Following DC Vaccinations

Patient no	Post I vaccination	Post II vaccination	Post III vaccination
2	Grade 0: itching at the site of vaccination	Grade 0: itching at the site of vaccination	NONE
7	NONE	Grade 1: fever chills and rigor due to urinary tract infection	Grade 0: vague abdominal discomfort and vomiting
10	NONE	NONE	Grade 1: 99F fever. The patient had higher ALP levels at the time of recruitment but lower than twice the upper limit so she fit the eligibility criteria. The levels persistently remained high throughout the study

*Only three patients developed adverse reactions to the DC vaccination. The nature and grade of toxicity is described below. The other patients did not develop any such symptoms following vaccination

Figure 5. X-ray Images of a Regressing Lung Metastasis in Patient 3. a) Lung metastatic lesion detected at patient enrolment (indicated by black arrow); b) and c) show progression of lesion after vaccination (5c. Was taken a year after vaccination); d) Shows complete regression of lesion three years after vaccination and 1.5 years after cis-platin chemotherapy

progression of her cancer and deterioration of her renal condition because of which DTH testing could not be done. Patient 14 dropped out of the trial just after receiving the first dose of vaccine.

Most of the arm I and II patients did not show any appreciable change in their disease status and were found to have disease progression during follow up. Patient 9 (arm III) had stable disease at follow up while Patient 3 (also in arm III) who was vaccinated in November 2004, had achieved local disease control at the primary site during her six month review after the third dose but showed progression of her lung metastatic lesion. She was advised symptomatic treatment in view of the progression of her lung metastasis in 2005. In Dec 2007, when updating the records we called the patient's relative telephonically to inquire about the patient's condition and were informed that she was fine. She was asked to be reviewed at the Institute. The patient presented to the Institute two years after her last follow up and clinically and radiologically she was found to be free of disease in the right lung (Figure 5). On enquiry, she revealed that she had received four injections of chemotherapy

with cis-platin between March and June 2006, a year and a half after the third dose. Examination of the cervix showed vaginal adhesions and she was considered to have achieved complete clinical remission. She continues to be disease free nearly eight years after treatment with cis-platin.

Two patients in arm II whose DC were not primed with tumor antigen responded with a positive DTH reaction. These patients responded to even the smaller tumor lysate dose (104). The survival status of some of the patients who were lost to follow up was determined by either letters written to them several times or telephonically while we could not ascertain the status of others.

DC vaccination improved proliferation response and IFN-gamma secretion post vaccination

One patient (Patient 3) showed improved proliferation of lymphocytes after the third vaccination (Figure 6) but the increase however was not significant (paired two tailed t test =0.06). No significant proliferation responses were seen in any of the other patients. Comparing arm II and arm III patients' responses also did not show significant difference. Secretion of IFN- γ was higher in post vaccination samples of 3/4 arm III patients and 2/5 arm II patients (Table 3). Although no significant difference between patients of the two arms could be ascertained, we observed that patients 9 and 12 of arm III who did not show a DTH positivity showed an IFN- γ response and patient 7

Table 3. IFN-Gamma Secretion in Vaccinated Patients

Patient no.	Pre vaccination (%)	Post third vaccination (%)
Patient 1	32	17.6
Patient 2	12	47.6
Patient 3	17.5	3.2
Patient 4	<1	<1
Patient 5	30.2	19.4
Patient 6	19.2	9.9
Patient 7	23.7	7
Patient 8	4.5	8.5
Patient 9	18.6	26.9
Patient 10	19.8	29.8
Patient 11	5.4	28.1
Patient 12	4.2	19.3

*Percentage of CD3+ cells secreting IFN-gamma before and after vaccination in each patient

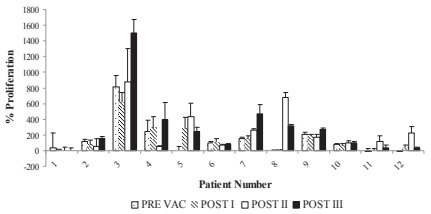

Figure 6. Proliferation Assay Using Autologous PBMC of Vaccinated Patients. Fifty thousand PBMC obtained before and after each dose of vaccination were monocyte depleted and stimulated with tumor lysate for 5 days (ratio 1:1). Unstimulated cells were taken as the baseline to determine percentage of proliferation which was measured using a colorimetric assay. Patient 3 showed robust proliferation responses post vaccination although the response was not strictly significant (p=0.06)

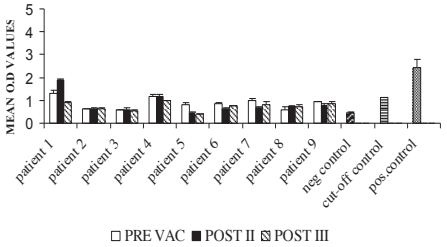

Figure 7. Anti-Nuclear Antibody ELISA. Anti-nuclear antibody ELISA was done using plasma samples from 9 patients obtained before vaccination (PRE VAC), after the second (POST II) and the third vaccination (POST III) as indicated above. Negative, cut off and positive controls are indicated above. Values were considered positive only if they were above the cut off control

(arm II) who had a robust DTH response did not produce higher levels of the cytokine, post vaccination. Patient thirteen could not be evaluated for immune response due to lack of tumor cells for the assays. Punch biopsy samples could not be obtained from all patients after the vaccine administration. Only three patients consented and hence immunohistochemistry was performed in those patients alone. Patient 9 who did not show any infiltration of CD8+ cells in the pre vaccination biopsy showed 30% CD8+ T cell infiltration in the post vaccination biopsy sample which was considered significant. But post vaccination biopsy samples could be obtained only from Patient 1, 2 and 9 hence the levels could not be compared evenly. Other markers like CD19, 56 and 45RO did not show any increase.

DC vaccination did not generate auto-immune responses in vaccinated patients

None of the nine patients evaluated had any elevation of auto antibody levels after the third vaccination although patient 1, who was in arm I (saline only) showed slight elevation of ANA levels (Figure 7) in the plasma obtained before the second dose. Although this value was slightly above the cut-off, it quickly dropped to well below the cut-off in the day 35 sample.

Discussion

This was the first study in India to use dendritic cells as therapeutic vaccines against HPV induced cervical cancer in a phase I clinical trial, with toxicity as its primary endpoint. Dendritic cell based therapeutic approach has now been applied as an adjuvant for the treatment of several types of cancers such as lymphomas (Hsu et al 1996), myelomas (Curti et al., 2007), brain cancers (Ardon et al., 2012) and pediatric malignancies (Dohnal et al., 2007) with some degree of success. Since no uniform standardised protocol has emerged, we developed a protocol and initiated a phase I trial. This trial was approved by the Institutional Ethical Committee and the Drug Controller General of India.

Fourteen patients enrolled in the trial and eleven of them received DC vaccinations. Of the eleven patients, six received DC matured with inflammatory cytokines alone while five received antigen primed mature DC. Three

other patients received saline only. A lower dose of DC was initially given but was later increased to one million DC, injected three times, at an interval of two weeks. One patient received a single dose of antigen loaded DCs and then opted out of the trial for reasons unknown.

A previous study showed that antigen loaded and cryopreserved mature DC were as potent as freshly loaded and matured DC (Thumann et al., 2003) providing an alternative to frequent blood sample collection for DC cultures. Since this approach was more practical, we used cryopreserved DCs for the study after appropriate testing for sterility.

Although different routes of vaccination exist, when given intranodally, the most frequent side effects so far reported to DC vaccines are systemic flu-like symptoms with fever or painful swelling of the injected lymph node whereas with intradermal injection only itching and erythema were the most commonly observed reactions that regressed within 48-72h in most cases. Additionally previous studies involving DCs and other vaccines have shown that the intradermal route is more physiologic with the injected cells migrating efficiently to the regional nodes (Reinhard et al., 2002, PATH, WHO review Dec-2010). Hence the intradermal route was chosen for our study. Itching and mild erythema were observed in two patients of our study but the others did not have any such complaints.

Some studies using immunotherapeutic adjuvants report that prior to inducing tumor rejection, there is immune cell infiltration leading to an increase in tumor size appearing as disease progression (Marchand et al., 1995). Even in our study we initially saw an increase in the responder's metastatic lesion and even though she received chemotherapy, this patient's disease free status nearly eight years later points to an immune system with active surveillance against tumor antigens.

DC vaccine trials especially those in glioma patients report massive intra tumoral infiltration of CD8+ T cells in several patients who received multiple DC vaccines (Liau et al., 2005). As we could not obtain a post vaccination biopsy from all our patients, it was not possible to ascertain the infiltration status in most of them especially, the patient who responded, but a biopsy from another patient who received lysate primed DC showed significant infiltration post vaccination.

Some biological agents that are used in immunotherapy may need longer time or booster doses for sustained and productive benefits. Dendritic cell vaccines may as well fall in this category when used against tumors. A study using DCs against glioma had shown that cytotoxic T cell activity may develop even twelve weeks (Yu et al., 2001) after vaccination while recently another reported CD8+T cell and IFN-γ responses in melanoma patients after they were given multiple DC vaccines loaded with a cocktail of synthetic peptides including MART-1 (Melanoma antigen recognized by autologous T cells-1) (Okoshita et al., 2012). Ardon et al. (2012) also noted that DC-based tumor vaccines in booster doses improved the six month progression free and median overall survival of newly diagnosed glioma patients who underwent surgery and concomitant chemoradiotherapy. For sustained stimulation of the immune system through multiple vaccinations, leukapheresis may be the best strategy. This process may yield large numbers of DCs for booster doses but since we did not get Institutional ethical committee clearance for the procedure we were only able to generate DC that were sufficient for three vaccination doses.

In a previous study (de Vries et al., 2003), patients with advanced melanoma were treated using both immature and mature DC with KLH as the adjuvant. They noted that none of the patients who received immature DC showed a positive DTH response while almost all the patients injected with mature DC developed a positive DTH which confirmed that DC maturation is a pre-requisite for vaccination. Although following vaccination, we did not expect any specific immune reaction especially to the unprimed mature DC two of the patients who responded to tumor lysates in DTH test were in arm II and responded to even the lower dose. This affirms the fact that even DC matured in the absence of antigen with cytokines alone may be active stimulators of the immune system.

Immunotherapeutic approaches especially DC vaccines have been used in combination with surgery and chemotherapy as a treatment modality in glioblastoma with standard treatment succeeded by vaccination. But a study conducted in 2004 (Wheeler et al., 2004) showed that vaccination or chemotherapy alone could not elicit significant tumor regressions whereas chemotherapy following DC vaccination significantly improved the mean survival of patients (p=0.04). Although most of our patients did not show any objective clinical response, one of the patients in arm III (patient 3) with a prominent lung metastasis, showed an excellent response to cis-platin chemotherapy given nearly a year after the third DC vaccine dose. She continues to be disease free for nearly 8 years now. Reports of an excellent complete response in metastatic cervical cancer are rare. A review on chemotherapy for metastatic and/or recurrent cervical cancer (Scatchard et al., 2012) found that in two randomized control studies, the median survival of patients after single agent cis-platin chemotherapy for metastatic disease was 17 (Alberts et al., 1987) and 13 (Cadron et al., 2005) months respectively. It is therefore interesting to speculate as to whether the DC vaccine had a contributory role in this patient achieving a sustained disease free status. The hypothesis put forward is that the mature antigen loaded DC vaccine induces an immune response as shown by the proliferation assay and DTH response. However, the immune response is unable to prevent the disease progression. The use of chemotherapy kills cells and releases tumor antigens acting as a booster dose. This could help mount a robust memory response in addition to the chemotherapeutic effect, effectively eliminating the tumor and extending a sustained anti tumor immune surveillance. This model therefore could have implications with regard to sequencing of treatment.

We plan to conduct a phase II trial in the near future with tumor lysate primed dendritic cells as adjuvants for HPV induced cervical cancer patients of stage IIIB along with concurrent chemo radiation as standard treatment.

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