

High-risk human papilloma virus infection decreases the frequency of dendritic Langerhans' cells in the human female genital tract

Rafael Jimenez-Flores, Rene Mendez-Cruz, Jorge Ojeda-Ortiz, Rebeca Muñoz-Molina, Oscar Balderas-Carrillo, Maria de la Luz Diaz-Soberanes, Serge Lebecque, Sem Saeland, Adrian Daneri-Navarro, Alejandro Garcia-Carranca, Stephen E. Ullrich and Leopoldo Flores-Romo

Summary

Dendritic cells (DC) are often arranged in planar layers in tissues with high antigenic exposure, such as skin and mucosae. Providing an *en face* view, this arrangement optimizes *in situ* analysis regarding morphology (even of individual dendrites), topographic distribution (regular/clustered) and quantification. The few reports on human genital DC usually utilize single markers and conventional sections, restricting immunolabelling only to cell parts sectioned by the cut. To better assess DC *in situ*, we labelled epithelial sheets, prepared from fresh cervix biopsies, with antibodies to major histocompatibility complex (MHC)-CII, CD1a and Langerin, revealing (with each of these markers) a dense DC network in a planar-like, regular distribution. Using the hybrid capture system to detect the high-risk mucotropic human papilloma virus (HPV) group, 16 positive and five negative women were studied and the results were compared between these groups. DC frequency per area was substantially reduced (to $\approx 50\%$ for the three markers) in samples from all HPV-infected patients compared with samples from controls. Unlike HPV⁻ samples, Langerin⁺ DC in HPV⁺ cervix exhibited a highly accentuated dendritic appearance. We believe this to be the first study using these three DC-restricted markers (Langerin, CD1a and MHC-CII) in cervical epithelial sheets from high-risk HPV⁺ donors and also the first study to demonstrate the morphological and quantitative changes triggered by high-risk HPV infection. Cervical DC reduction in early, premalignant high-risk HPV infection might represent viral subversion strategies interfering with efficient antigen handling by the immune system's peripheral sentinels, the DC, perhaps hampering appropriate recruitment and subsequent development of effector (cytotoxic) T cells.

Introduction

Dendritic cells (DC) constitute a system of highly specialized antigen presenting cells (APC) that function as sentinel posts of the immune system, especially in peripheral non lymphoid tissues that have a high antigenic exposure, such as the skin and mucosae.¹ Under stimuli of diverse

origin (e.g. microbial, inflammatory or cytokines), DC are induced to migrate from their peripheral locations into the nearest lymphoid tissues where they will usually localize in the T cell areas.^{2,3} It is now clear that, apart from the original antigen (Ag) or inflammatory stimuli, signals like chemokines and chemokine receptors will function to facilitate and guide DC trafficking.⁴ This complex process

Abbreviations: Ab, antibody; Ag, antigen; APC, antigen presenting cell; CIN, cervical intraepithelial neoplasia; CTL, cytotoxic T lymphocyte; DAB, diaminobenzidine; DC, dendritic cell; HPV, human papilloma virus; mAb, monoclonal antibody; MHC, major histocompatibility complex; RLU, relative light units; SS, saline solution; TZ, transformation zone.

culminates in the appropriate delivery of processed Ag to lymphocytes and the initiation of efficient immune responses. As T cells need Ag transportation and processing, their dependence on DC is an absolute requirement to efficiently initiate their functions as effector cells, and this holds for both CD4⁺ and CD8⁺ (cytotoxic) T lymphocytes.⁵

Although DC have been described in the human female genital tract, especially in the cervix, these reports are scarce regarding healthy samples and are at least contradictory concerning human papilloma virus (HPV) infected tissues. Some researchers have found no changes, some have reported an increased DC frequency, while others have found decreased DC numbers.⁶⁻⁸ This might be explained by the fact that the samples used may have been from HPV infected subjects at different stages of the disease, or taken from different histological areas in each person.

Potential subversion strategies of micro organisms to evade effector T cell responses could be either to attack the T cells directly (human immunodeficiency virus, for instance), or to interfere with the APC (like *Mycobacterium tuberculosis*),⁹ on which T cell responses are strictly dependent.

Human cervical cancer is the first recognized by the World Health Organization (WHO) as being 100% attributable to an infectious agent, the high risk HPV.^{10,11} More than 80% of the cases of this neoplasia occur in developing countries where good programmes for screening, prevention or treatment are still awaiting implementation. Nevertheless, excellent research, over a time period of 10–20 years, has been carried out on several aspects of this malignancy, including the clinical evolution and the classifications of this protracted disease; likewise, the complete genomes of many HPVs are known.^{12,13}

There are, however, gaps in the understanding of this cancer of infectious origin and the potential interactions with the local immune system in the female genital tract. For instance, by necessity, most patients with cervical cancer are first seen and investigated when the disease has already evolved to a neoplastic stage, and perhaps this has made the research in the early phases of the HPV infection less abundant.

Likewise, studies of regional immune responses in the healthy, intact human female genital tract are rather scarce. Although it is assumed that HPV enters through micro abrasions and infects basal epithelial stem cells using mechanisms and molecules not yet elucidated,¹⁴ the type of immune responses (if any) occurring locally in the affected tissues during the early phases of HPV infection are virtually unknown. Moreover, it seems that there is a poor inflammatory response under HPV infection during the early cervical intraepithelial neoplasia (CIN) phases.^{10,11}

At a systemic level it is known that antibodies (Abs) can be induced to L1 or to E7 viral proteins. However, in the case of L1, Abs might take months or years to

appear,¹⁰ while antibodies to E7 are uncommon before the onset of the invasive disease, despite the fact that E7 protein might have been expressed during the previous 10 or 20 years. In any case, however, these Ab responses are clearly non protective because the disease continues to develop.¹⁵

Cellular immunity to HPV, as assessed mainly by *in vitro* T cell reactivity, has been more difficult to demonstrate, and reports are contradictory.¹⁰ However, despite the infectious origin of this neoplasia, and despite the chronicity of the infection, it seems that the cytotoxic T lymphocytes (CTL) of a substantial proportion of women have been found to be unresponsive to HPV.⁷ Faulty local (epithelial) Ag handling and processing has been proposed to account for this.¹⁰ There is one report, nonetheless, indicating that delayed type hypersensitivity (DTH) to E7 correlates well with the resolution of CIN.¹⁶

At least part of the seemingly faulty (humoral and cellular) immune responses towards HPV might be explained by the fact that HPV infects basal epithelial stem cells, but for replication it apparently requires that these cells undergo differentiation. During differentiation, these cells transit from the basal cellular stratum towards the luminal (external) side where the mature virions are finally produced and released. Moreover, HPV does not have a lytic phase; rather, it seems to induce cell proliferation, thus lacking a viraemic stage during the infection, when dissemination and presentation of viral Ags would occur.¹⁷ Therefore, both systemically and in the local infected tissue, no or scarce possibilities of useful contacts exist between the HPV and the elements of the immune system.^{10,17}

In an attempt to initiate *in situ* studies on the regional immune responses during the early stages of human female infection with high risk HPV, we selected two groups of Mexican women. This segregation was based on several, carefully evaluated, criteria, including a specialized clinical examination, laboratory tests, cytopathology, and the positivity or negativity for a set of high risk HPV, according to the hybrid capture method. Epithelial sheets from fresh cervical biopsies from these women were evaluated for the presence, phenotype and frequency of DC, using three well established DC markers.

Materials and methods

Selection of patients and control subjects

The protocol for this study was first reviewed and approved by the local ethical board of the Regional Hospital of Pachuca, Mexico's Health Secretariat, Hidalgo state. Non pregnant women who, during a period of 13 months (2001–02), were referred for a colposcopic evaluation to the specialized gynecological services at the Regional Hospital because of an abnormal Pap smear,

were considered for enrolment in the study. These women were carefully evaluated, which involved undertaking a complete gynaecological examination, including colposcopy, cytobrush, Pap smear and aceto white testing. Only those women who, by all criteria, were categorized as having low grade disease (CIN I) and with lesions large enough to provide biopsies, were considered, asked to participate in the study and requested to provide written, informed consent. CIN I was chosen because is an early premalignant lesion, usually in the so called transformation zone (TZ) of the cervix, and associated with manifestations of HPV infection, such as koilocytosis. Of a total of 796 women attending the first visit to this service during the period of October 2001 to October 2002, 23 were preselected on the basis of the results of the clinical evaluations mentioned above. Of these, after the clinical, laboratory and histopathology evaluations, 16 were selected on the basis of their positivity for high risk HPV, according to the results obtained following analysis by the hybrid capture system kit (Digene, Gaithersburg, MD). Five women, undergoing hysterectomy for benign conditions, were included as controls, according to negative results when analysed by the criteria listed above, including gynaecological evaluations, the cytology results and the hybrid capture system kit. A further step was included in which the data of the selected patients were analysed by an expert pathologist at the hospital.

Immunohistochemistry of conventional tissue sections of cervix (HPV⁻ and HPV⁺ samples)

Cervical biopsies were taken from the TZ in CIN I patients and from equivalent areas in control (hysterectomy) women. Samples were then placed in sterile Eppendorf vials (Eppendorf North America, New York, NY, USA) with 500 µl of endotoxin free sterile saline solution (SS) and maintained on ice until use; a small piece was then cut and placed in tissue tek (Sakura Finetek, Torrence, CA, USA) and kept frozen at -70° until immunolabelling procedures took place. Once 5 µm tissue sections had been prepared, fixed with cold acetone for 7 min, and blocked with Universal Blocking Solution (BioGenes, San Ramon, CA), primary Abs were used at optimal dilutions, which were previously determined. Ab to MHC CII (DR) (Dako, Carpinteria, CA) was used at a dilution of 1 : 50, and Ab to L1 (Pharmingen, San Diego, CA) was used at a dilution of 1 : 50. This Ab recognizes L1 from HPV16, although it may cross react with HPV 33, but not with HVP 6 or HPV 11. Both Abs were left to react overnight at 4°. After thorough washing of the sections, procedures were then exactly the same as for epithelial sheets undergoing immunohistochemistry. A secondary Ab, peroxidase conjugated horse anti mouse immunoglobulin (Vector, Burlingame, CA), was added in a 50 µl volume and incubated at room temperature for

1 hr; after three more washes, bound Abs were revealed using diaminobenzidine (DAB) as chromogen, observed as a dark brown colour. The reaction was stopped by washing in running water, mounting media was added and the slides were coverslipped before analysis.

Preparation of epithelial sheets from human cervix

Once the biopsies were taken, they were placed in a sterile Eppendorf vial containing 500 µl of endotoxin free sterile SS, and placed on ice until their preparation in the laboratory, which usually took less than 3 hr. Once in the laboratory, samples were cut into two main portions; the smallest was placed in tissue tek and frozen until sectioning for immunohistochemistry, as described below, while the largest portion was placed, lumen side up, in an enzymatic cocktail containing collagenase dispase, for 15–20 min at 30°. After this incubation period, the epithelial layer was separated by gentle traction with fine forceps, stretched very carefully and washed three times in sterile SS containing 1% bovine serum albumin (SS + 1% BSA), fixed in 2% paraformaldehyde for 20 min and then washed extensively before initiating the immunohistochemistry procedures.

Immunohistochemistry of cervical epithelial layers to assess DC in situ

Each epithelial layer was divided into small pieces in order to have at least three portions for testing each Ab. The following monoclonal antibodies (mAbs) were used: anti DR (Dako) at a dilution of 1 : 50; anti CD1a (Dako) at a dilution of 1 : 50; and anti Langerin (LIR, Dardilly, France) at a dilution of 1 : 50. For each set of experiments, an isotype matched, irrelevant control Ab was also included. These Abs were applied overnight at 4°, in a final volume of 50 µl per well, for each set of cervical epithelial sheets. Then, epithelial sheets were washed three times in SS + 1% BSA, and secondary Ab (peroxidase conjugated horse anti mouse immunoglobulin) (Vector, Burlingame, CA) was added in 50 µl of SS + 1% BSA and incubated at room temperature for 1 hr. After washing three times in SS + 1% BSA, bound Abs were revealed using DAB as chromogen, observed as a dark brown colour. Once the reaction was stopped, epithelial layers were stretched to make them as flat as possible, immunomount solution was added, and the slides were coverslipped and analysed using a Carl Zeiss microscope. At least 20 different fields were evaluated for each different portion of epithelial sheet obtained.

Hybrid capture system to identify the group of high-risk HPV

A small piece of the cervix sample was placed in Digene hybrid capture transport medium (Digene Diagnostics

Inc., Silver Springs, MD), following the manufacturer's instructions. Samples were then incubated in denaturation reagent to produce single stranded DNA. The HPV RNA probe cocktails for high risk HPV were added with either control or test samples and allowed to hybridize at 65° for 1 hr. The hybridized DNA RNA was then placed into tubes coated with anti hybrid immunoglobulin, and immobilized hybrids were then incubated with alkaline phosphatase tagged Abs. After incubation and subsequent washing, the Lumi Phos detection reagent was added to each tube, the tubes were incubated for 30 min at room temperature, after which they were read using a lumino meter. Each set of samples was run with the positive and negative controls provided by the hybrid capture kit. Results are expressed as relative light units (RLU) and represent the mean of triplicate readings for each sample.

Data analysis

Mean values and standard deviations were calculated for numerical variables. The Mann Whitney test was performed to compare the DC density with positive markers between study groups (HPV⁺ and HPV⁻ controls). All results were viewed as significant if $P < 0.05$. Bivariate correlation between DC density with the age of patients, and DC density with the study group, were analysed using Spearman's rho non parametric test. In order to evaluate both independent variables as predictors of DC density, we used a linear regression by enter method. The data were analysed using the software Statistics Professional Social Science (SPSS[®]) version 10 for Windows[®].

Results

Histology of conventional tissue sections and identification of MHC-CII (DR)⁺ cells in normal cervix

We first evaluated the normal histological appearance of the cervix region, especially around the TZ. In Fig. 1(a), the epithelium (upper part) is readily distinguishable from the stroma (lower part). Likewise, cells labelled for MHC CII are clearly apparent, especially in the basal cells of the epithelium (arrows in Fig. 1b,c). Occasionally, the cut permits that one of these MHC CII⁺ cells might resemble a DC (arrow in Fig. 1c), although their morphology in the sections does not allow such a precise distinction to be made.

Cervical epithelial sheets as an appropriate tool for *in situ* assessment of genital tract DC

By means of enzymatic treatment, we successfully adapted, to freshly taken cervix samples, the approach of epidermal sheet separation. The epithelial layers obtained from cervix

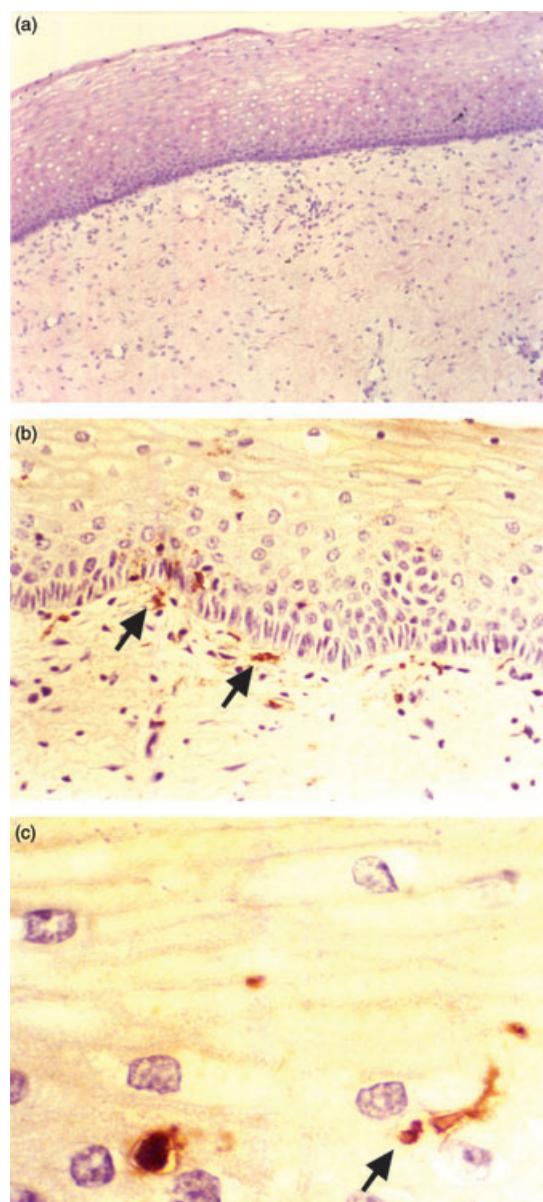


Figure 1. Basic histology and major histocompatibility complex (MHC) CII (DR)⁺ cells in conventional tissue sections of human papilloma virus (HPV) negative cervix. Cervix samples obtained from high risk HPV negative women were frozen, and conventional tissue sections were stained with haematoxylin (a) and with a monoclonal antibody to MHC II (DR) (b, c). The epithelium is clearly distinguishable from the underlying stroma (a; $\times 10$ magnification). In this section, bound MHC II antibody was revealed with peroxidase and diaminobenzidine (DAB), as a brown colour (b, c). Brown MHC CII⁺ cells are clearly evident around the lower stratum of the cervix epithelium (arrows in panel b; $\times 20$ magnification). In some of these cells, a dendritic appearance was revealed (arrow in panel c; $\times 40$ magnification).

tissue upon this treatment were, indeed, useful for examining the DC morphology. Indeed, by using this approach it was possible to evaluate not only individual DC, each

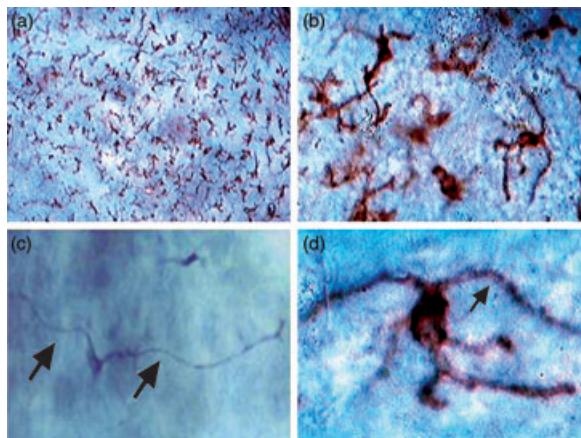


Figure 2. Cervical epithelial sheets reveal cells with prominent dendritic morphology and a regular pattern of distribution. Fresh cervix biopsies were subjected to enzymatic treatment to separate epithelial layers in order to perform immunohistochemistry with antibodies to CD1a (a, b, d) and major histocompatibility complex (MHC) CII (DR) (c). A dense network of strongly positive cells with notorious dendritic appearance is clearly revealed in epithelial sheets from cervix tissue. Likewise, a regular pattern of distribution is readily observable (a). Not only the morphology of individual, complete cells can be assessed, but also single dendrites in each DC (c and d, arrows). (a) $\times 10$ magnification; (b) and (c) $\times 40$ magnification; (d) $\times 100$ magnification.

one complete, but also the extension and the arrangements of single dendrites in a given cell (arrows in Fig. 2c,d). This was also a very convenient means of assessing whether DC showed any peculiar pattern of distribution in the cervix, which would not be possible to determine using conventional tissue sectioning. For instance, a dense network of DC in a planar like arrangement was revealed with each of the Abs used (Fig. 2a; CD1a staining). Indeed, the DC topography shown with these three markers revealed that DC are not randomly scattered along the cervix epithelium, but instead are regularly distributed (Fig. 2a), like DC in the skin. In fact, both the morphology and the regular pattern of distribution resembled very much that of the epidermal Langerhan's cells (LC), and was manifested through each of the three Abs used: DR, CD1a and Langerin (Fig. 3a c). However, as in the skin, cervical DC were more obviously dendritic when revealed with MHC II DR and CD1a than with Langerin, which, perhaps owing to the staining of the soma, did not provide such a prominent dendritic appearance, but instead delineated well the DC body (Fig. 3c).

HPV⁺ CIN-I versus HPV⁻ (control) samples

By means of the hybrid capture method, a small portion of freshly obtained tissue was used to assess the presence of high risk HPV (nine types of mucotropic HPV). It was clear that most samples of the clinically preselected

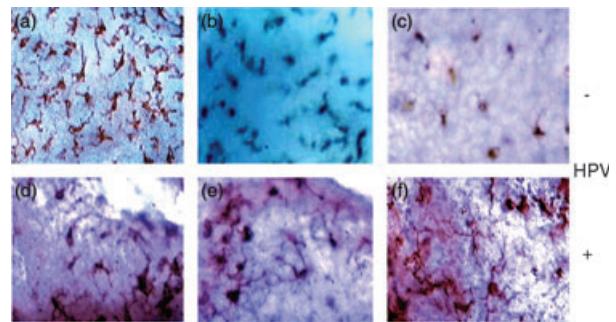


Figure 3. Dendritic cells (DC) in cervical epithelial sheets of human papilloma virus negative (HPV⁻) and human papilloma virus positive (HPV⁺) patients. Epithelial layers of cervix samples obtained from HPV⁻ and HPV⁺ cervical intraepithelial neoplasia I (CIN I) patients were used to assess the morphology, distribution and frequency of DC with antibodies to CD1a (a and d), major histocompatibility complex class II (MHC II) (DR) (b and e) and Langerin (c and f); (a) (f), $\times 40$ magnification. Apart from quantitative and morphological changes, the regular pattern of DC distribution was also modified in the HPV infected samples (d-f). The morphology of Langerin⁺ cells was clearly altered in HPV⁺ sheets (f) compared with HPV samples (c).

patients (CIN I stage) provided a very strong signal for high risk HPV in the luminometer readings, and only a few (four) gave a weak, but positive, signal (Fig. 4, black bars). It was also evident that for the samples taken from control patients, readings were clearly negative in this system (Fig. 4, white bars), thus correlating well with all the criteria (clinical, laboratory tests and cyto pathology) used for preselecting both groups.

Samples from HPV⁺ cervix express, *in situ*, abundant L1 protein of HPV16 and koilocytosis

Frozen sections of fresh cervix biopsies from HPV⁺, CIN I grouped women, were subjected to immunohistochemistry using Abs to L1 of high risk HPV16. Numerous epithelial cells from these HPV⁺ samples were found to strongly express L1 HPV16 protein, and the positivity increased towards the lumen (Fig. 5b-d). Basal cells of the epithelium, at the lower stratum, and also cells in the underlying stroma, were L1 negative (Fig. 5b). The cells termed koilocytes were also evident in the tissue (arrows in Fig. 5c,d), thus verifying the cellular manifestations typical of these early epithelial lesions during HPV infection.

Reduction of DC frequency in cervix layers of HPV⁺ patients

Once the immunolabelling of DC in cervical epithelial sheets had been established, we performed a comparison between DC in epithelial layers obtained from control, HPV negative cervix tissue and samples from high risk

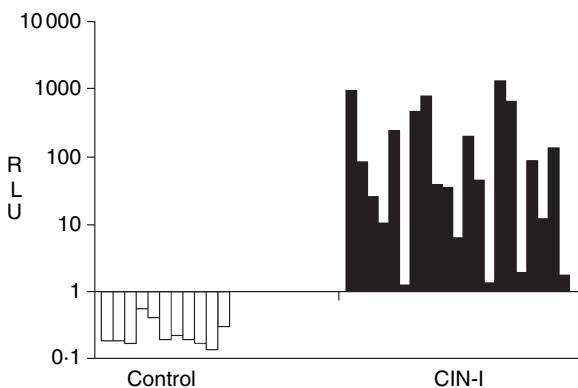


Figure 4. Cervical intraepithelial neoplasia (CIN) I samples, but not control samples, are positive for high risk human papilloma virus (HPV). Fresh tissue samples from cervix were evaluated for the presence of the high risk HPV by using the hybrid capture system. Women ($n = 16$) were first preselected using multiple criteria (clinical, laboratory tests and cyto pathology), and samples were tested for the presence of DNA of the high risk HPV group. Control samples were from hysterectomy performed for benign conditions in $n = 5$ women, showing normal results using the same clinical and cyto pathology criteria. Control samples (left side of the figure) were all negative for the presence of high risk HPV, while the 16 CIN I preselected samples were positive for high risk HPV (right side of the figure). Most CIN I samples were strongly positive. The results are expressed as relative light units (RLU) and represent the mean of triplicate readings for each sample.

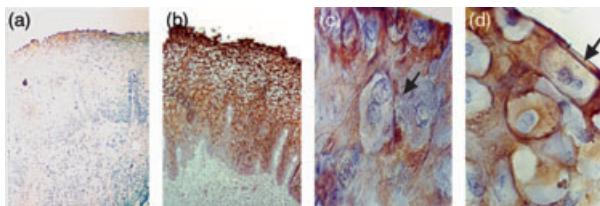


Figure 5. L1 protein expression and koilocytosis in human papiloma virus (HPV)⁺ cervical intraepithelial neoplasia I (CIN I) cervix. Cervix samples from CIN I grouped women were subjected to immunohistochemistry using antibodies to the L1 protein of HPV16. Abundant cells in the tissue strongly express the L1 protein, especially towards the upper stratum of the epithelium (b-d), while basal epithelial cells and stromal cells are negative (b). Likewise, koilocytosis, a typical cytopathic response of early infection, is easily observed in the HPV infected epithelium (arrows in c and d). (a) HPV⁺ epithelium labelled with an irrelevant isotype matched control antibody. (a) $\times 10$ magnification; (b) $\times 20$ magnification; (c) and (d) $\times 100$ magnification.

HPV⁺ women. As shown in Fig. 3(d-f) and Fig. 6, DC were less numerous in samples prepared from HPV⁺ women than in epithelial sheets obtained from non infected cervix samples. In fact, at $\approx 50\%$, DC reduction was similar for all three markers (Fig. 6). Statistically significant differences between the HPV⁻ and the HPV⁺ groups were found for all three markers examined. The corre-

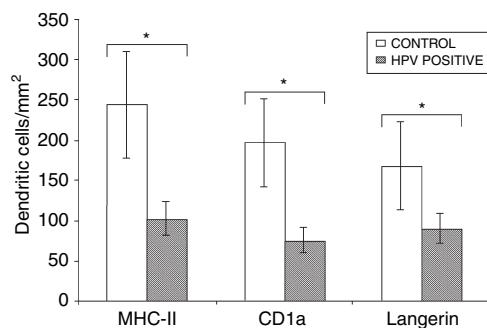


Figure 6. High risk human papilloma virus (HPV) infection reduces the number of dendritic cells (DC) in human cervix. Epithelial layers from freshly obtained cervix samples were used to quantify the DC frequency per area in all tissue samples obtained from both control HPV (white bars) and HPV⁺ (grey bars) women. Assessment was performed for each of the three markers [major histocompatibility complex class II (MHC II), CD1a and Langerin], and the results represent the mean value plus standard error of all quantifications performed, at least in triplicate, on each sample from all the women examined. (* $P < 0.001$).

tion between the DC frequency with the age of patients was low and similar for all markers, at ≈ 0.40 . The correlation between the DC density with the variable study groups was 0.59 for MHC II, 0.61 for CD1a and 0.51 for Langerin. Standardized coefficients obtained with linear regression were as follows: 0.64 for MHC II when group variable was the predictor, and 0.02 for age; 0.69 for CD1a with group variable as the predictor, and 0.01 for age; and 0.51 for Langerin with group variable as the predictor, and 0.12 for age.

Altered morphology of Langerin⁺ DC in HPV⁺ cervix

Langerin staining in epithelial sheets from cervix depicted mainly the cell soma of DC, rather than providing a clearly delineated dendritic morphology like that observed with MHC II⁺ or CD1a⁺ DC. It was interesting therefore to find that Langerin staining revealed a prominent dendritic appearance (Fig. 3f) only in HPV⁺ samples, perhaps indicating signs of DC activation *in situ*.

Discussion

Although there are a number of reports of DC in the HPV infected human female genital tract, especially in the cervix, these appear to be contradictory because some describe no changes, some have found a decreased number of DC and others have seen increased DC numbers.¹⁸⁻²¹ Various reasons have been suggested for these discrepancies.

- (1) The precise histological locations where the biopsies were taken might have been different.

- (2) The disease stages of the HPV infected samples examined may have been different.
- (3) Most studies used only single, but different, markers to identify DC: most used S 100; others used were MHC CII²² and ATPase. Fewer have used CD1a,²³ and we know of no studies that have used the Lang erhans' cell restricted marker, Langerin. Furthermore, to the best of our knowledge there are no descriptions of these three DC markers (MHC CII, CD1a, Lang erin) evaluated together.
- (4) The methodological approach used to perform these evaluations should also be taken into account. As far as we know, researchers have used mostly conventional tissue sections not only to identify DC, but also to quantify them. However, we believe that for accurately assessing DC, at least three different criteria (evident dendritic morphology, topographic distribution within tissues and, perhaps more important for these studies, DC quantification) should be used. The use of cervical epithelial sheets is more appropriate than conventional tissue sectioning. We believe this because, if DC are arranged in a planar like layer (which they seem to be in mucosae and skin) in a rather uniform distribution, only those parts of a DC body traversed by the cut will be visualized during immunolabelling. In contrast, epithelial sheets, by providing an *en face* view of each complete cell, are a very convenient means of simultaneously assessing morphology (even at the level of a single cell and a single dendrite, arrows in Fig. 2c,d), the pattern of DC distribution and optimizing their quantification per tissue area. Moreover, it is also important to consider that cervical epithelial sheets, not undergoing the process of paraffin embedding and not being frozen, but freshly prepared and immediately fixed, are more likely to provide a closer approximation of how the tissue and the cells might be in reality. In fact, it was readily observed that DC in the cervix epithelium are not randomly scattered, and they do not seem to be clustered in certain areas along the epithelium. Instead, they were found to exhibit a rather regular and uniform pattern of distribution, resembling very much that of cutaneous LC in epidermal sheets (Fig. 2a). Moreover, this regular pattern of distribution was revealed with all of the three DC markers used (CII, CD1a and Langerin), although with different morphological appearances, especially for Langerin⁺ cells.

We are interested in studying *in situ* the local, regional immune responses in the female genital tract, especially during the development of the early premalignant stages of HPV infection. We have therefore chosen to initiate these studies with women positive for the group of viruses associated with cervical carcinoma – the high risk

HPV group – and we chose to study only patients categorized as having CIN I. Although this stage represents a premalignant lesion appearing early during HPV infection, these lesions express viral proteins. Usually these lesions will be associated with peculiar cellular manifestations, termed koilocytosis (a cytopathic consequence of the early viral infection), which appear in the TZ. Indeed, koilocytes were readily observed in the cervical epithelium of our HPV⁺ samples examined (arrows in Fig. 5c,d). Furthermore, positivity was also manifested for the L1 viral protein (but not for the E7 viral protein; data not shown) (Fig. 5) in the epithelium of seven of the samples examined, being stronger towards the lumen. However, in these same samples in which a strong positivity for the viral protein L1 was revealed, no labelled cells exhibited an apparent dendritic morphology (Fig. 5b). Furthermore, no L1 positive cells were seen in the area where MHC II⁺ cells were located (Figs 1b and 5b). This suggests that high risk HPV infection, at least in an early premalignant stage, such as CIN I, does not reach or target these local APC; more detailed research is needed to elucidate this. In fact, it is known that mature HPV virions are produced at the most external cell layers of the cervix and released into the lumen. HPV progeny are shed into the environment with epithelial squamae, therefore precluding useful contacts with cells of the local or the systemic immune system, somehow making the host immunologically unaware of the infection.^{6,7}

We do not have a precise explanation for the decrease of DC found in the HPV infected cervix, although several possibilities exist, including: reduction of the DC (or their immediate precursors) colonizing this area; increased exiting or traffic of the normally resident cells; or promoted DC death. We believe that the last possibility, although possible, is less likely to occur because HPV is known to induce proliferation rather than cell death, at least in epithelial cells.⁶ We are currently examining these possibilities. The mean age of the HPV⁺ CIN I group was 32.5 years and that of the control HPV⁻ hysterectomy group was 42.5 years. Because of ethical constraints it was not feasible to biopsy healthy women of younger age with no medical justification. However, the correlation between DC density and age was low and similar for all markers. Additionally, the standardized coefficients of multivariate analysis indicate that the observed changes in DC frequency were not related to age, but to the HPV infection status. Moreover, in another epithelium, such as the skin, DC frequency has been reported to be substantially reduced in the elderly,²⁴ not increased. If there were any parallel between these two epithelia, the skin report indirectly supports our findings in the cervix because the decreased DC frequency was found in the younger (HPV⁺ CIN I), and not in the older (hysterectomy, control), age group.

Regarding the potential meaning and consequences of the low DC frequency found in the cervix samples of all patients infected with high risk HPV, as DC are the most peripheral sentinel cells of the immune system and are crucial for delivering appropriately processed antigenic information to T cells, this may represent one of many viral subversion stratagems given that the three markers used to identify the DC (MHC CII, CD1a and Langerin), are all importantly involved in Ag capturing, processing and presentation. Conceivably, a consequence of this could be a more permissive microenvironment for the HPV to initiate and establish infection within the regional tissue. On the one hand, local DC deficiency is likely to impede efficient T cell recruitment into this microenvironment. On the other hand, local DC deficit might also hamper the efficient ferrying of Ag into regional lymph nodes, thus further interfering with the development of effective cytotoxic T cell responses. Another important fact to consider is that not only the quantity of DC was decreased in the HPV infected cervix; HPV might also affect the early process of DC activation because, in these same samples where MHC II⁺, CD1a⁺ and Langerin⁺ cells were readily identified, we were unable to demonstrate DC expressing CD40 (not shown), a molecule well known to be induced by different stimuli during the normal process of DC activation and maturation. It is thus intriguing that precisely within the cervical tissue microenvironment where the HPV infection naturally occurs, there are fewer DC and those remaining might not be activated and may not be actually infected. Viral infection during these early stages (CIN I) might therefore interfere with establishing a local tissue microenvironment appropriate to the subsequent development of efficacious T cell responses, possibly facilitating, in this way, the progression of these early lesions into carcinomas. According to some,⁶ HPV infection might thus create a localized immunodeficient microenvironment, especially in the TZ. Perhaps all of these different contributing factors may help to explain the reports on the intriguing findings that apparently many patients lack CTL reactivity, even after long periods (months or years) of documented HPV infection.^{6,7} More research is certainly required, first in order to obtain a better picture of the early responses of the local immune system and the potential viral subversion strategies and, second, to try to develop better therapeutic or prophylactic approaches for this cancer of infectious origin.

Acknowledgements

The authors wish to thank all the team of the Cellular Immunology Laboratory (CINVESTAV IPN), the personnel from the Cervical Pathology and Colposcopy Unit, Pachuca Regional Hospital, and Dr Rosa Maria Chi R (Gyneco Obstetrics Hospital Tlatelolco, IMSS) for all help provided. This research was performed, in part, under

grants from UNAM PAPIIT IN 221799 & Conacyt. A. Daneri, A. G. Carranca, and L. Flores Romo are SNI members.

References

- Banchereau J, Steinman R. Dendritic cells and the control of immunity. *Nature* 1998; **392**:245 52.
- Cumberbatch M, Dearman RJ, Griffiths CE, Kimber I. Langerhans cells migration. *Clin Exp Dermatol* 2000; **25**:413 8.
- Cumberbatch M, Brushan M, Dearman RJ, Kimber I, Griffiths CE. IL 1 beta induced Langerhans' cell migration and TNF alpha production in human skin: regulation by lactoferrin. *Clin Exp Immunol* 2003; **132**:352 9.
- Sato N, Ahuja SK, Quiñones M, Kostecki V, Reddick RL, Melby PC, Kuziel WA, Ahuja SS. CC chemokine receptor (CCR)2 is required for Langerhans cell migration and localization of T helper cell type 1 (Th1) inducing dendritic cells. Absence of CCR2 shifts the *Leishmania major* resistant phenotype to a susceptible state dominated by Th2 cytokines, B cell outgrowth, and sustained neutrophilic inflammation. *J Exp Med* 2000; **192**:205 18.
- Alexander M, Salgaller ML, Celis E, Sette A, Barnes WA, Rosenberg SA, Steller MA. Generation of tumor specific cytolytic T lymphocytes from peripheral blood of cervical cancer patients by *in vitro* stimulation with a synthetic human papillomavirus type 16, E7 epitope. *Am J Obstet Gynecol* 1996; **175**:1586 93.
- Giannini SI, Hubert P, Doyen J, Boniver J, Delvenne P. Influence of the mucosal epithelium microenvironment on Langerhans' cells. Implications for the development of squamous intraepithelial lesions of the cervix. *Int J Cancer* 2002; **97**:654 9.
- Connor J, Ferrer K, John P, Kane B, Goldberg JM. Evaluation of Langerhans cells in the cervical epithelium of women with cervical intraepithelial neoplasia. *Gynecol Oncol* 1999; **75**:130 5.
- Morris H, Gatter KC, Stein H, Mason DY. Langerhans' cells in human cervical epithelium: an immunohistological study. *Br J Obstet Gynaecol* 1983; **90**:400 11.
- García RGS, Pedroza GA, Aguilar LD, Orozco EH, Lambrecht BN, Estrada GI, Flores RL, Hernández PR. Airways infection with virulent *Mycobacterium tuberculosis* delays the influx of dendritic cells and expression of costimulatory molecules in mediastinal lymph nodes. *Immunology* 2004; **112**:661 8.
- Frazer IH. Prevention of cervical cancer through papillomavirus vaccination. *Nat Rev Immunol* 2004; **4**:46 54.
- Bosch FX, Muñoz N. The viral etiology of cervical cancer. *Virus Res* 2002; **89**:183 90.
- Mougin C. *et al.* Human papillomaviruses, cell cycle and cervical cancer. *J Gynecol Obstet Biol Reprod (PARIS)* 2000; **29**:13 20.
- Bosch FX, Lorincz A, Muñoz N, Meijer CJ, Shah KV. The causal relation between human papillomavirus and cervical cancer. *J Clin Pathol* 2002; **55**:244 65.
- McMillan NA, Payne E, Frazer IH. Expression of the alpha 6 integrin confers papillomavirus binding upon receptor negative B cells. *Virology* 1999; **261**:271 9.
- Lehtinen M, Pawlita M, Dillner MJ *et al.* Evaluation of antibody response to human papillomavirus early proteins in women in whom cervical cancer developed 1–20 years later. *Am J Obstet Gynecol* 2003; **188**:49 55.

- 16 Hopfl R, Heim K, Christensen N *et al.* Spontaneous regression of CIN and delayed type hypersensitivity to HPV 16 onco protein E7. *Lancet* 2000; **356**:1985 6.
- 17 Schiller JT, Davies PP. Delivering on the promise. HPV vaccines and cervical cancer. *Nat Rev Microbiol* 2004; **2**:343 7.
- 18 Rasbridge SA, Jenkins D, Tay SK. A histological and immuno histological study of cervical intraepithelial neoplasia in relation to recurrence after local treatment. *Br J Obstet Gynaecol* 1990; **97**:245 50.
- 19 Matthews K, Leong CM, Baxter L, Inglis E, Yun K, Backstrom BT, Doorbar J, Hibma M. Depletions of Langerhans cells in human papillomavirus type 16 infected skin is associated with E6 mediated down regulation of E cadherin. *J Virol* 2003; **77**: 8378 85.
- 20 Morelli AE, Sananes C, Di Paola G, Paredes A, Fainboim L. Relationship between types of human papillomavirus and Langer hans' cells in cervical condyloma and intraepithelial neoplasia. *Am J Clin Pathol* 1993; **99**:200 6.
- 21 Vayrynen M, Syrjanen K, Mantyjarvi R, Castren O, Saarikoski S. Langerhans cells in human papillomavirus (HPV) lesions of the uterine cervix identified by the monoclonal antibody OKT 6. *Int J Gynaecol Obstet* 1984; **22**:375 83.
- 22 Sajic D, Patrick AJ, Rosenthal KL. Mucosal delivery of CpG oligodeoxynucleotides expand functional dendritic cells and macrophages in the vagina. *Immunology* 2005; **114**:213 24.
- 23 Mota F, Rayment S, Chong S, Singer A, Chain B. The antigen presenting environment in normal and human papillomavirus (HPV) related premalignant cervical epithelium. *Clin Exp Immunol* 1999; **116**:33 40.
- 24 Flores Langarica A, Calderon AJ, Becerril GM *et al.* Decreased frequency of cutaneous dendritic cells in elderly subjects. *Allergy Clin Immunol Int* 2005; **17**:152 5.