

## Cellular Subsets and Epithelial ICAM-1 and HLA-DR Expression in Human Papillomavirus Infection of the Vulva

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Cryostat sections of 20 clinical condylomata of the vulva induced by human papillomavirus and 5 normal control biopsies were examined using immunohistochemistry. The results indicated that in vulvar papillomavirus infection the intraepithelial Langerhans' cells showed abnormal morphology and a significantly lower density than controls. CD1a positive Langerhans' cells were also observed in dermis of condylomata, suggesting an abnormal epithelial traffic of dendritic cells. T lymphocytes with a mean CD4/CD8 ratio of 0.25 and a mean density of  $267 \pm 59$  cells/mm<sup>2</sup> of epithelial section were the main cellular infiltrate in vulvar papillomavirus infection. Most of the T cells were HLA-DR negative. Those condylomata with moderate to severe mononuclear infiltrate showed leucocyte function antigen 1 positive T cells forming small clusters in the lower epithelial half around the ICAM-1 positive keratinocytes. Vulvar warts also showed epithelial areas with overlapped ICAM-1 and HLA-DR expression. Scattered T gamma-delta and B lymphocytes, macrophages and NK cells were observed among the cells of the dermal infiltrate of vulvar condylomata. **Key words:** Langerhans' cells; Lymphocytes; HPV.

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Local immune response plays an important role during human papillomavirus (HPV) infection (1–4). A mild to severe mononuclear infiltrate, composed mainly by T lymphocytes, is frequently present both in non-regressing and spontaneously regressing skin warts (1, 5, 6).

During normal and pathological conditions, cell adhesion molecules (CAMs) are involved in the regulation of the epithelial leucocyte transit, allowing interactions among lymphocytes, endothelial cells and keratinocytes (7). Within the CAMs, the subfamily of integrins termed LeuCAMs includes three adhesion molecules mainly expressed by leucocytes: leucocyte function antigen 1 (LFA-1), macrophage activation antigen 1 (Mac-1) and P 150,95 (7). One known ligand for LFA-1 is the intercellular adhesion molecule 1 (ICAM-1). In skin diseases associated with mononuclear infiltrate, ICAM-1 is expressed by endothelial cells and by keratinocytes (7). It is also known that the ICAM-1 expression observed in endothelia and keratinocytes can be upregulated in vitro by cytokines (8–10).

Even though clinical and histopathological features of HPV infection of the vulva are well documented, little is known about the modulation of CAMs and the immunophenotype of the local cellular immune response generated by the virus. In

the present study we investigated the expression of CAMs and the changes observed in T cell subsets, B lymphocytes, macrophages, natural killer cells and Langerhans' cells (LCs) in vulvar condylomata induced by HPV.

### MATERIAL AND METHODS

#### Handling of samples

Twenty women ranging between 20 and 54 years of age with clinical vulvar condylomatous lesions and previous histopathological diagnosis of HPV infection were selected for the present study. Under vulvoscopic examination and after topical application of 5% trichloroacetic acid for 5 min, 2 adjacent biopsies were taken from vulvar acetowhite areas of each patient; one of the biopsies was fixed in buffered formalin for conventional histology and the second was snap-frozen in liquid nitrogen, using an isopentane interphase, and stored at  $-70^{\circ}\text{C}$  until use for immunohistochemistry.

As normal controls, 5 paired biopsies taken from vulva of a group of 5 women similar in age and with normal vulvoscopic appearance were also processed for histology and immunohistochemistry.

The purpose of this study as well as any associated risk was explained to patients and controls and written consent was obtained from them before specimen collection.

#### Immunoenzymatic staining

Frozen sections from normal and pathologic samples were cut at 6–8 microns in thickness. The sections were mounted on clean slides coated with polylysine, air-dried overnight, fixed in acetone for 10 min at room temperature and then immersed in PBS containing 3% hydrogen peroxide for 30 min to inhibit endogenous peroxidase activity. The sections were incubated for 30 min with human group AB serum diluted 1:20 to block Fc receptors of mononuclear cells, and then stained by the peroxidase-antiperoxidase (PAP) complex method. Briefly, the slides were sequentially incubated overnight with the primary monoclonal antibody (mAb), followed by rabbit anti-mouse Ig (1:40) and monoclonal PAP immune complexes (1:200). After rinsing, peroxidase activity was detected with 3,3'-diaminobenzidine tetrahydrochloride (DAB) as developing agent. After extensive washing with distilled water, the tissue sections were counter stained with hematoxylin, dehydrated and mounted.

The detection of the capsid antigen of HPV was performed by indirect immunoperoxidase labelling on frozen sections. Briefly, after blocking endogenous peroxidase activity and Fc receptors as described, the sections were incubated with a rabbit anti-HPV serum (1:50) overnight at  $4^{\circ}\text{C}$ . Then swine anti-rabbit Ig antisera conjugated with peroxidase (1:40) were added and the slides were incubated for 30 min at room temperature. After rinsing, peroxidase activity was detected using DAB and the tissue sections were counterstained, dehydrated and mounted.

Negative controls were incubated with PBS or unreactive murine mAb of IgG 1 and IgG 2a isotypes in order to rule out Fc receptor-mediated binding, unspecific absorption of antibodies or endogenous peroxidase activity.

#### Antibodies and antisera

mAbs used in the present study are listed in Table I. They define the following cellular subsets or epitopes: NA 1/34; LCs and immature

Table I. *mAbs and the heterologous antisera used in this study*

Agent	Epitope	Isotype	Source
<i>mAbs (clones)</i>			
NA1/34	CD1a	IgG2a	A Mc Michael, Oxford, U.K.
L243	HLA-DR	IgG1	American Type Culture Collection
UCHT1	CD3	IgG1	P Beverley, London, U.K.
MT310	CD4	IgG1	Dakopatts. Code N° M716
UCHT4	CD8	IgG2a	P Beverley, London, U.K.
F11	Gamma/Delta TCR	IgG1	A Moretta, Genova, Italy
HD37.1	CD19	IgG1	Dakopatts. Code N° M740
MY13	CD54	IgG1	IV International Workshop on Human Leucocyte Differentiation Antigen
MHM24	CD11a	IgG1	IV International Workshop on Human Leucocyte Differentiation Antigen
T199	CD56	IgG1	Dakopatts. Code N° M852
EBM11	CD68	IgG1	IV International Workshop on Human Leucocyte Differentiation Antigen
P6/38	Peroxidase-anti-Peroxidase Soluble Complex	IgG1	Sigma. Code N° P-2416
<i>Heterologous antisera</i>			
Rabbit anti-HPV			Dakopatts. Code N° B580
Peroxidase-conjugated swine anti-rabbit immunoglobulins			Dakopatts. Code N° M-8019
Goat anti-mouse polyvalent immunoglobulins			Sigma. Code N° M-8019

thymocytes; L243: major histocompatibility antigen class II (expressed by B lymphocytes, activated T lymphocytes, LCs, macrophages and activated keratinocytes); UCHT1: mature T cells; MT310: helper/inducer T cells, a subset of macrophages and LCs; UCHT4: cytotoxic/suppressor T cells; F11: gamma/delta T cells; HD37.1: mature B cells; MY13: adhesion molecule ICAM-1 (ligand for LFA-1); MHM24: adhesion molecule LFA-1 (ligand for ICAM-1) expressed by all leucocytes; T199: natural killer cells and EBM 11: macrophages.

#### *Immunophenotyping of serial sections*

In order to demonstrate coexpression of different molecules on the mononuclear infiltrate and on the corresponding epithelial areas, serial sections were made of each sample. The serial sections were incubated with anti HLA-DR, anti-CD1a, anti-CD3, anti-CD4, anti-CD8, anti-CD11a, anti-gamma/delta T cell receptor (TCR), anti CD19, and anti-CD54. This method allowed us to compare the same histologic area in adjacent sections.

#### *Histopathological parameters of HPV infection*

The histopathological diagnosis of vulvar condyloma was based on the presence of the following features: acanthosis, koilocytosis, binucleation, degenerative nuclear alteration induced by the virus (pyknosis, irregular nuclear membrane and irregular distribution of the chroma-

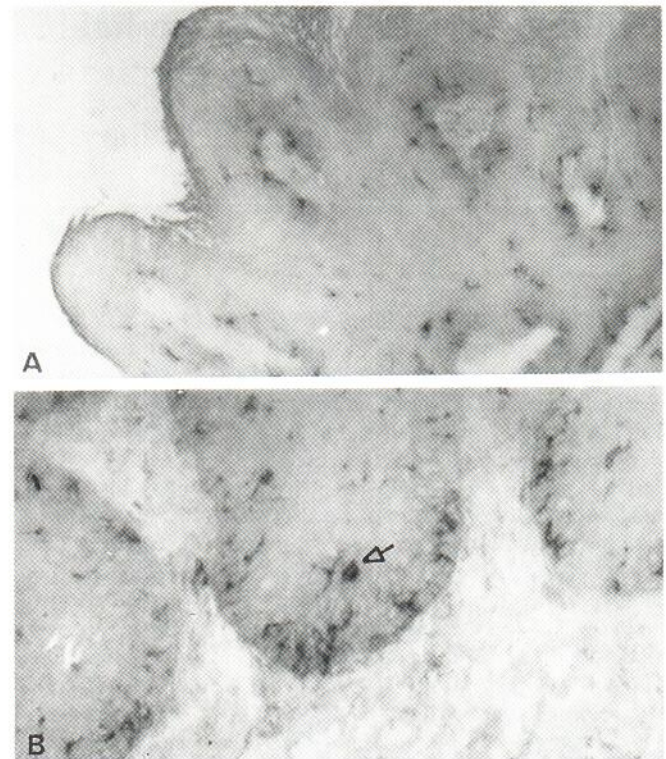


Fig. 1. LCs in vulvar HPV infection: (A) Abnormal distribution of intraepithelial LCs in a condyloma acuminatum of vulva (CD1a-PAP $\times$ 100); (B) Arrow indicates an LC, round in shape, with short and non-arborized dendrites in another area of the same condyloma (CD1a-PAP $\times$ 400).

tin), parakeratosis and basal cell hyperplasia (11). In condylomata acuminata, acanthosis and hyperkeratosis expanded the lesion surface into papillary projections. In spiculate condyloma the hyperkeratosis produced projections towards the surface of the lesion.

Table II. *Subsets of intraepithelial T lymphocytes in frozen sections of vulvar condylomas*

Case N°	CD3 <sup>a</sup>	CD4 per CD3 (%)	CD8 per CD3 (%)	CD4/CD8 ratio	Gamma/Delta <sup>a</sup>
1	51	25	75	0.33	0
2	175	11	86	0.13	0
3	148	5	91	0.06	0
4	127	25	71	0.35	0
5	98	8	92	0.09	1
6	1200	19	77	0.25	3
7	360	24	74	0.32	0
8	250	16	80	0.20	0
9	85	25	73	0.34	0
10	80	28	71	0.39	1
11	712	14	84	0.17	0
12	156	17	81	0.21	0
13	272	10	84	0.11	0
14	280	19	80	0.24	2
15	250	15	83	0.18	0
16	80	29	70	0.41	1
17	130	17	81	0.21	2
18	250	20	75	0.27	0
19	272	30	69	0.44	0
20	360	24	70	0.34	1

<sup>a</sup> Counted as positive cells per mm<sup>2</sup> of vertical epithelial section.

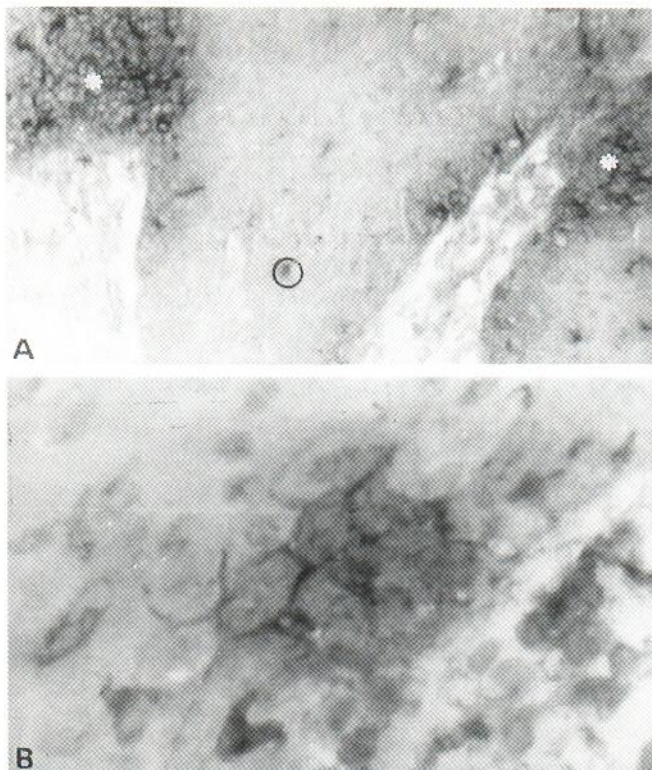


Fig. 2. (A) ICAM-1 expression on two small epithelial areas of a vulvar condyloma (white asterisks). Notice that some LCs are also positive; one of them is encircled (ICAM-1-PAP- $\times 400$ ); (B) Chicken wire pattern of ICAM-1 positivity due to membrane immunostaining of keratinocytes in vulvar condyloma (ICAM-1-PAP- $\times 1000$ ).

#### Cell count

Density of the positive cells was determined as the number of cells per  $\text{mm}^2$  of vertical histological section within the epidermis, papillary and reticular dermis. All positive cells in two different sections for each marker were counted in controls and pathological samples by using a microscope with a calibrated grid in the eyepiece.

The intensity and extent of ICAM-1 and HLA-DR staining on endothelium of dermal vessels were scored according to the method described by Griffiths et al. (10). Briefly, four high-power fields of the strongest stained areas were assessed per section.

Intensity of immunostaining was graded on a scale from 0 to 5: 0, no staining; 1, minimal; 2, mild; 3, moderate; 4, intense; 5, maximal staining. The extent of the staining was graded by the relative percentage of positive endothelial cells in the postcapillary venules on a scale from 0 to 4: 0, no staining; 1, up to 25%; 2, 25% to 50%; 3, 50% to 75%; 4, 75% to 100% of positive cells. Therefore, the maximal score was 9, obtained by adding the intensity assessment (0 to 5) and the diffuseness of staining (0 to 4).

#### Statistical analysis

All values were expressed as means  $\pm$  sd. For statistical evaluation, non-parametric statistics were used by means of the Wilcoxon rank-sum test (12). A *p* value of less than 0.05 was regarded as significant.

## RESULTS

### Histopathological findings

All the samples taken from vulvoscopically abnormal areas fulfilled the histopathological criteria for vulvar condyloma induced by HPV and were not associated with vulvar intraepithelial neoplasia.

The HPV capsid antigen was detected in 11 out of the 20

samples (55%). Hematoxylin and eosin staining confirmed the presence of a chronic inflammatory infiltrate in 18 out of the 20 samples (90%). The infiltrate was mild in 4, moderate in 9, and severe in 5 cases.

The control vulvar biopsies showed no abnormalities and did not express HPV antigen.

### Langerhans' cells

Most of the intraepithelial CD1a positive LCs showed abnormal features in vulvar condylomata. The cells were irregularly located along the lower half of the epithelium (Fig. 1A). They were round in shape with short, non-arborized and stunted dendritic prolongations (Fig. 1B). In 8 condylomata (40%) a few CD1a positive LCs were detected in the dermis in close apposition to the subepithelial mononuclear infiltrate. The mean density of intraepithelial CD1a positive LCs in vulvar warts was  $138 \pm 65$  cells/ $\text{mm}^2$ , which was significantly lower than that observed in normal controls ( $321 \pm 57$  cells/ $\text{mm}^2$ ) ( $p = 0.001$ ). In condylomata, an intraepithelial LCs subset was also strongly positive for ICAM-1 (60% of the LCs population based on the mean count of CD1a positive LCs performed on serial sections). A small number of LCs (11%) were also positive for LFA-1.

In normal controls, CD1a positive LCs were regularly distributed along the basal and suprabasal layers of the epithelium. They showed long and well arborized prolongations and were negative for ICAM-1 and LFA-1. No CD1a positive cells were present in the underlying dermis.

### Lymphocytes

In condylomata, intraepithelial lymphocytes were predominantly of T cell type and were localized in the lower epithelial half. In cases with severe inflammatory infiltrate, the T cells were observed in small intraepithelial clusters around keratinocytes.

The mean density of intraepithelial CD3 positive cells was  $267 \pm 59$  cells per  $\text{mm}^2$  of tissue section, which was significantly higher than that observed in controls ( $27 \pm 8$  cells/ $\text{mm}^2$ ) ( $p < 0.05$ ). The density of the different T cell subsets is depicted in Table II. Intraepithelial CD8 positive cells were

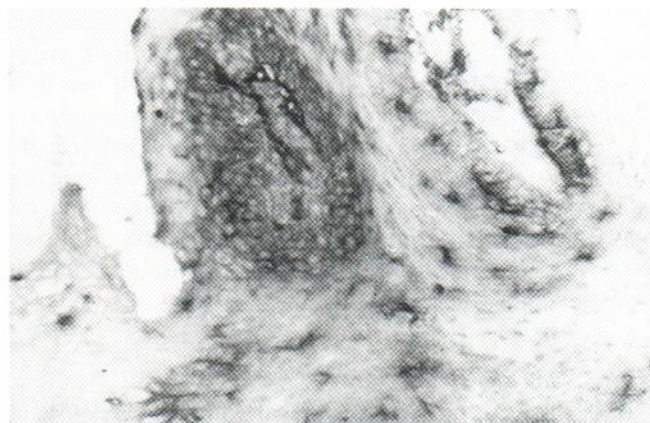


Fig. 3. Focal HLA-DR expression in keratinocytes and LCs in condyloma (HLA-DR-PAP- $\times 400$ ).

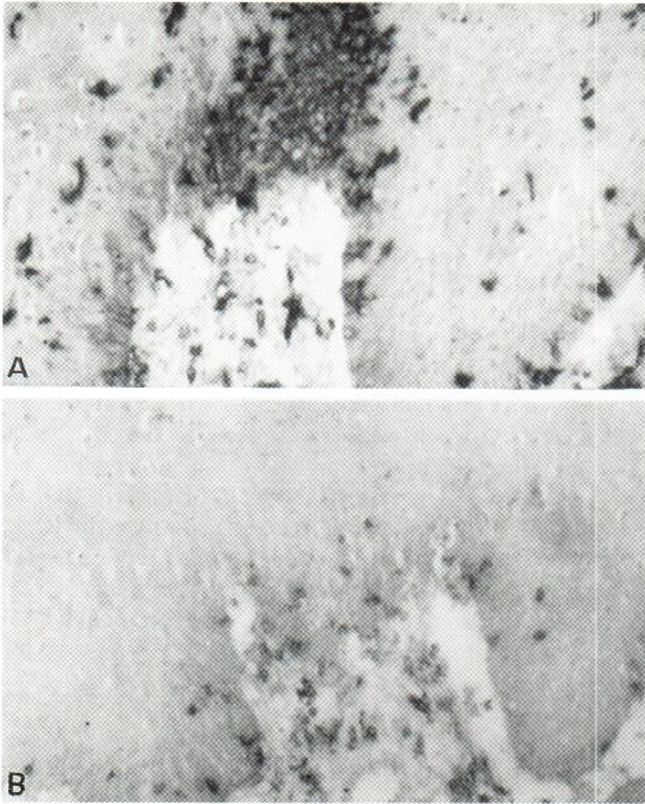


Fig. 4. Serial sections of a condyloma demonstrated: (A) focal ICAM-1 positivity on vulvar epithelium infected by HPV; and (B) a small cluster of CD3 + mature T lymphocytes infiltrating the same zone (ICAM-1 and CD3-PAP- $\times 400$ ).

significantly higher than CD4 positive cells ( $p < 0.001$ ), with a mean CD4/CD8 ratio of 0.25. A similar CD4/CD8 ratio (0.34) was counted in normal controls.

Interestingly, in all the condylomata studied, most of the intraepithelial lymphocytes were HLA-DR negative.

In vulvar HPV infection the lymphocyte infiltrate was mainly located in the dermis in close apposition to the epithelial basement membrane or surrounding the postcapillary venules. The mean count of dermal T cells was  $716 \pm 138$  cells/mm<sup>2</sup>, which was significantly higher than in normal controls ( $339 \pm 53$  cells/mm<sup>2</sup>) ( $p < 0.05$ ). The CD4/CD8 ratio was higher than 1 in 10 samples and lower than 1 in the remaining warts. Except for the lymphocytes localized close to endothelial cells, the dermal lymphocytes were HLA-DR negative.

T cells expressing the gamma-delta TCR comprised only 5% of the intraepithelial lymphocytes of the control vulvar biopsies and were absent in the underlying dermis. They were not an important component of the T cell population of the local immune reaction against HPV infection (Table II).

B cells were rarely detected in the epithelium and dermis of biopsies from normal controls and condylomata.

*ICAM-1 and HLA-DR expression on epithelial cells*

Vulvar condylomata were associated with scattered foci of ICAM-1 and HLA-DR positive keratinocytes (Figs. 2 and 3). The expression of these molecules on the cell membrane, with

a slight cytoplasmatic staining, was responsible for the "chicken wire" pattern observed (Fig. 2B). The ICAM-1 and HLA-DR positive areas were located in the lower half of the epithelium, including the basal, the parabasal and the stratum spinosum. Serial sections of the same epithelial areas confirmed an overlapped expression of both markers. In samples with severe mononuclear infiltration, ICAM-1 positive epidermal areas were associated with small intraepithelial clusters of LFA-1 positive lymphocytes (Fig. 4).

No ICAM-1 or HLA-DR staining of keratinocytes was observed in control samples.

*ICAM-1 and HLA-DR expression on endothelial cells*

Vulvar warts showed an intense ICAM-1 and HLA-DR expression on endothelial cells of the superficial and deep vascular plexus of dermis. Those cases with severe or moderate cell infiltration organized around dermal vessels (transformed into high endothelial venules: HEV) showed the strongest and most diffuse endothelial staining for ICAM-1 and HLA-DR antigens (Fig. 5). In control biopsies, the relative expression of these markers was lower than that detected in endothelial cells of condylomata (Fig. 5).

*Natural killer cells and macrophages*

Both macrophages and natural killer cells were preferentially localized close to the basement membrane and free in the connective tissue of the papillary dermis both in controls and condylomata. Although vulvar warts showed a number of macrophages and natural killer cells higher than controls (data not shown), the differences were not significant.

DISCUSSION

The decrease in the number of LCs in HPV infection and a presumed diminished local immune surveillance have been postulated as the cause of persistent viral infection in certain tissues, such as the uterine cervix (13-16). The persistent infection by the HPV types associated with evolution to intraepithelial neoplasia was suspected of being a cofactor responsible for malignant transformation in those tissues with a high incidence of cancer (13-16).

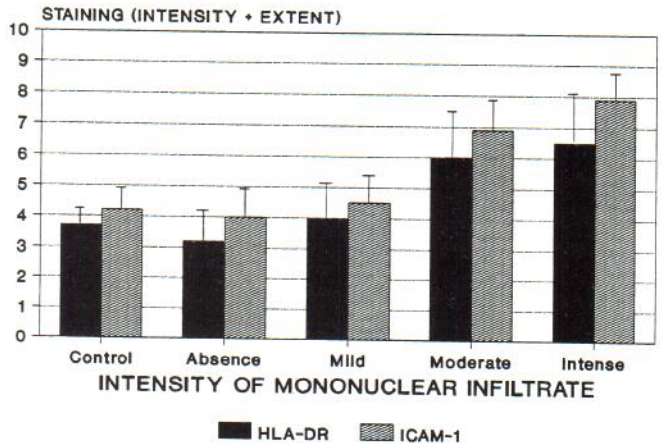


Fig. 5. ICAM-1 and HLA-DR staining of vascular endothelium in vulvar condylomas.

The reduction in dendritic cell density, along with an abnormal cellular morphology, has also been described in human warts induced by HPV in skin, uterine cervix, vulva, penis and oesophagus (13–23). Curiously, vulvar and penile warts, which have a lower incidence of malignant transformation compared with the cervix but are infected by the same types of HPV, also showed a significant decrease in the number of LCs (1, 22). Moreover, we have recently demonstrated that in uterine cervix the warts induced by HPV types 16 and 18, which are associated with malignant transformation, showed a decrease in LC number similar to that caused by the HPV types 6 and 11 (24). All these data argue against an association between low number of LCs and progression of malignancy during genital HPV infection.

The reason for the partial depletion of LCs during HPV infection remains unknown. CD1a positive LCs were observed in the dermis of vulvar condylomas in close apposition to T lymphocytes. This unusual presence of CD1a+ LCs in the dermis could be explained by an increased transit of the LCs through the infected epithelium. Tagami & Thivolet also observed this phenomenon in spontaneously regressing and non-regressing flat warts of skin (1, 6).

During HPV infection LCs upregulated the expression of ICAM-1 and LFA-1. The expression of ICAM-1 was demonstrated in vitro in LCs undergoing differentiation into interdigitating dendritic cells (25). Combining these data, the induction of CAMs on LCs of condylomata might be considered a sign of intraepithelial cell maturation before LCs leave the epidermis towards the afferent lymph nodes.

In our study, the expression of HLA-DR on keratinocytes was only observed in cases with moderate or severe mononuclear infiltrate. In other skin disorders, the secretion of interferon (IFN) gamma by activated cells from the cellular infiltrate is associated with HLA-DR expression on keratinocytes (26).

During the local immune response to the HPV vulvar infection, the cell count demonstrated that T lymphocytes (mainly CD8) migrate into the epithelium. After that, T cells might attach to keratinocytes by means of LFA-1, as has been reported in skin cultures and in frozen-section cytoadherence assays (7, 27).

On the other hand, the molecule ICAM-1 (LFA-1 ligand), is expressed by keratinocytes in experimental conditions (such as induction by IFN-gamma) or in pathological conditions where activated T lymphocytes constitute a putative source of IFN-gamma (9, 10, 27).

Our study has demonstrated that HPV infection is associated with a low expression of ICAM-1 in keratinocytes on restricted epithelial areas of the vulvar epithelium. In those cases with moderate to intense cellular infiltrate, serial sections confirmed that these areas were associated with small intraepithelial clusters of LFA-1 positive T lymphocytes. The low number of activated T lymphocytes (based on the HLA-DR expression and putative responsibility for cytokine secretion) observed in the infiltrate might explain the few ICAM-1 positive epithelial areas detected in vulvar HPV infection.

Though the immunoperoxidase method used in the present

study was not quantitative, the semiquantitative analysis of the dermis demonstrated that the relative increase of ICAM-1 expression on endothelial cells was associated with the intensity of the mononuclear infiltrate. The upregulation of ICAM-1 endothelial expression might explain a better adherence of circulating leucocytes to the endothelium of the HPV-infected area and could allow the mononuclear infiltration of the tissue.

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