

## Counts and areas of S-100-positive epidermal dendritic cells in atypical molluscum contagiosum affecting HIV+ patients

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**Summary.** Molluscum contagiosum is a common and self-limiting viral infection, that in HIV+ patients courses as an opportunist affection with atypical clinical features. Impaired cell-mediated immune response could be involved in such atypical growth. We evaluated the density and area of Langerhans cells (LC) using S-100 immunohistochemistry in seven atypical molluscum contagiosum. LC density was quantified by three different methods using computer-assisted morphometry as well as estimating the relative area of LC with respect to epidermal area. Results were compared with two control groups (normal skin specimens and molluscum contagiosum affecting non-AIDS healthy patients).

We found a virtual absence of LC in areas of molluscum lesions affecting both HIV+ and non-AIDS patients. Likewise we observed an evident decrease in LC density in perilesional epidermis of atypical molluscum with respect to both control groups. Upon comparing the counts and areas, we observed that this reduction in LC count was statistically significant only when considering LC related to length of basement membrane in atypical molluscum with respect to normal skin specimens. Our finding of a reduced number of LC in the perilesional epidermis of HIV+ patients with atypical molluscum could explain the high frequency and clinical challenge of molluscum contagiosum in immunocompromised people. In spite of these results, further studies of LC kinetics and functions are required to precisely elucidate their role in the course of molluscum contagiosum in HIV+ patients.

**Key words:** Langerhans cells, Molluscum contagiosum, HIV infection, Immunohistochemistry, Computer-assisted morphometry

### Introduction

Molluscum contagiosum (MC) is a common viral infection of the skin that has traditionally been considered as a benign, asymptomatic and self-limiting affection (Myskowski, 1997). It is caused by the molluscum contagiosum virus (MCV), which is a DNA virus member of the poxvirus (poxviridae) family, and the sole member of the molluscipoxvirus genus (Senkevich et al., 1996).

Atypical lesions of MC (AMC), often attaining great size with multiple or disseminated skin lesions, that may mimic other cutaneous diseases, have been frequently described over the last two decades, affecting immunocompromised hosts and especially HIV-infected patients, suggesting that cell-mediated immunity plays a significant role in controlling MCV infection. AMC in HIV+ patients has now become a common infection in which the clinical course is marked by chronic lesions that are often recalcitrant to treatment and may become disfiguring (Cronin et al., 1996; Myskowski, 1997). AMC are often considered to be good markers of late-stage HIV disease with an inverse correlation between CD4 count and the number of molluscum lesions (Schwartz and Myskowski, 1992). However, besides this inverse ratio, little is known about the host-virus relationships that could explain the clinical challenge of MC in immunocompromised people.

Langerhans cells (LC) are a normal component of human epidermis and skin-associated lymphoid tissue, where they act as potent antigen-presenting cells (Breathnach, 1988). Various authors point out that LC may constitute an HIV target of infection (Stingl et al., 1990; Zambruno et al., 1991) which may contribute to the appearance of diverse dermatological conditions of opportunistic character, including MC, although some studies on LC density in HIV+ patients (Dreno et al., 1988; Nandwani et al., 1996) have shown conflicting results. Therefore, the evaluation of the density and area of LCs by means of computer-assisted morphometry appears to be of interest, in order to explain the high

frequency of MC in HIV+ patients and its atypical growth and evolution.

### **Materials and methods**

#### *Patients and skin biopsies*

A retrospective study was conducted in six HIV+ patients with AMC diagnosed in the University Hospital La Fe, Valencia. The clinical setting was a tertiary care referral center. These patients form part of a large HIV+ population that has been evaluated at our institution in recent years. All patients were HIV-seropositive by standard laboratory methods (enzyme-linked immunosorbent assay followed by confirmatory Western blot analysis). Most patients were involved in HIV+-related treatment protocols, and clinical data including anatomic sites, dimensions, numbers and clinical aspects of MC lesions as well as the occurrence of other opportunistic infections and CD4/CD8 counts were retrospectively obtained from the clinical records. In one patient two biopsies of two different MC lesions presented within an interval of a year were evaluated.

In all cases the AMC diagnosis of skin lesions was confirmed by biopsy. Briefly, skin specimens were fixed in 10% formaldehyde saline, embedded in paraffin, and stained with hematoxylin and eosin, PAS and Masson's trichrome for routine histopathological diagnosis.

#### *Immunohistochemical staining*

From the original paraffin blocks additional sections of 5  $\mu\text{m}$  of all skin specimens were cut, and immunohistologically stained using the three-step avidin-biotin immunoperoxidase method. Pretreatment with trypsin (0.1% in phosphate buffer) was made for 20 min. at 37 °C. Primary prediluted polyclonal antibody anti-bovine S-100 (Biomedica) was used at room temperature for 60 min. Negative controls were performed by omitting the primary antiserum.

#### *Control cases*

We designed two groups of control cases in order to evaluate and compare the density and area of LC. The first group consisted of five specimens from normal skin (one from the thigh and four from breast tissue) of healthy Caucasian non-AIDS patients (one male, four females) that were obtained from surgery for cosmetic reasons, or during surgical reduction procedures. These control subjects were selected by excluding those who had previously been exposed to factors known to diminish epidermal LC populations (Yu et al., 1994). These included concomitant psoriasis, treatment with immunosuppressive agents, corticosteroids, radiotherapy or high levels of ultraviolet light (such as sunlight exposure, sunbeds or PUVA therapy) within the previous month. The second group of control cases for LC morphometric evaluation consisted of three skin biopsies

of typical cases of MC, affecting well-known healthy non-AIDS patients and diagnosed in our hospital prior to 1980.

#### *Quantitative analysis of Langerhans cells*

Quantitative analysis of LC was performed using slides immunostained with S-100 protein both in the seven MC lesion affecting HIV+ patients and in the two control group samples. Only cells with a distinctive nucleus and at least one dendritic process were identified and counted as LC. To exclude most melanocytes, only positive cells above the basal layer were counted. In AMC cases (HIV+ patients) as well as in MC control cases (healthy non-AIDS patients), five high power fields (HPF) (x40 objective) were evaluated in the lesional area and also five HPF in the perilesional epidermis were counted. In the control normal skin specimens, only five HPF were counted, excluding areas around the hair follicles, taking into account the peculiar distribution of LCs in such locations (Moresi and Horn, 1997).

LC density in the epidermis was calculated by three different methods, as previously described (Bieber et al., 1988): a) LC number detected per mm length of epidermal surface; b) LC number observed per mm of basement membrane length; and c) LC number observed per square mm of epidermal area examined. In addition, we also calculated the surface area of LC in absolute value ( $\mu\text{m}^2$ ) and in relation to the total epidermal area (expressed in %), because it is known that a reduction in LC density may be compensated for by an increase in the mean cell volume (Yu et al., 1994). All results were calculated by means of the mean values of the counted cells and measurements found in the five fields examined. All measurements (lengths and areas) and quantitative analyses were performed using an Olympus® light microscope with a color charge coupling device video camera (CCD) connected to a Micro-Image® computer morphometry system, with a x40 objective (frame area for each captured image was 0.030948  $\text{mm}^2$ ).

#### *Statistical analysis*

The statistical evaluation of the results was performed by means of the SPSS-X statistical computer package (SPSS Inc, Chicago, IL) using the non-parametric two-tailed Mann-Whitney and Student's T tests. For all tests, a mean p-value of 0.05 or less was considered to be significant.

### **Results**

#### *Atypical molluscum contagiosum observations*

AMC infection in HIV+ patients affected six patients, four males and two females, ages ranging 6-55, mean age 34.5 years, with one patient biopsied twice

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within a year, and finally by studying seven biopsy cases of AMC. Two patients were homosexual males, three intravenous drug abusers (two males, one assumed to be a heterosexual male, and one female), and a girl, aged 6 years, with maternal-filial transmission, was also biopsied. Four patients were in advanced HIV infection stage, thus meeting AIDS criteria (II-C, III-C, IV-C, D and E stages). At the time of diagnosis of AMC, patients had a median CD4+ T-lymphocyte count of 54 cells/mm<sup>3</sup> (range 6 to 400) with a CD4+/CD8+ ratio between 0.86 and 0.02. In all cases the lesions were clinically multiple and/or disseminated, often affecting face, scalp, eyelids (Fig. 1), as well as submandibular and preauricular regions. In three cases there were giant lesions (diameter > 1 cm) and also in three cases lesions showed a cystic appearance. Clinically, three cases were initially misdiagnosed as epidermal cyst, dermal fibroma and basocellular carcinoma. The lesions were repeatedly treated with cryosurgery, but total destruction of all lesions was not achieved and new lesions appeared later.

Histological examination in all cases revealed a hyperplastic, cup-shaped invagination of the epidermis composed of multiple acanthotic lobules with intermediate and superficial keratinocytes containing characteristic intracytoplasmic Henderson-Patterson inclusion bodies. Molluscum bodies continued to move toward the epidermal surface and ultimately discharged into the central plug with presence of inclusion bodies and disintegrated stratum corneum. In three cases cystic structures compatible with an epidermoid cyst with MC

infection of the cystic wall (Fig. 2) appeared on light microscopic examination. The internal parts of cysts often contained corneal debris and molluscum bodies. Close to these cysts hairs with viral folliculitis often appeared, suggesting that the origin of the cysts were follicular. None of the cases showed associated dermal inflammatory infiltrate.

#### *S-100 immunostaining of epidermal dendritic cells and comparative LC quantification (AMC and control groups)*

The first control group corresponding to five normal skin samples immunostained with S-100 protein revealed a population of strongly positive dendritic cells within the Malpighi strata, identified as LC. A second population of weaker S-100 staining, with more rounded cells, was also present; these were almost exclusively contained within the basal layer and were probably melanocytes. Evaluating only the suprabasal and dendritic (at least with one dendritic process) S-100-positive epidermal cells in morphometric count as LC (Fig. 3), we found in this control group a mean value of  $284.84 \pm 36.95$  LC by mm<sup>2</sup> of epidermis (ranging between 177.78 and 338.60), representing the LC area  $0.83 \pm 0.10$  % of total epidermal area. On other hand, LC number with respect to mm of epidermal and basement membrane length was  $21.79 \pm 1.76$  and  $16.02 \pm 1.36$  respectively.

A difference of normal skin in all AMC cases analyzed, a virtual absence of S-100 epidermal dendritic

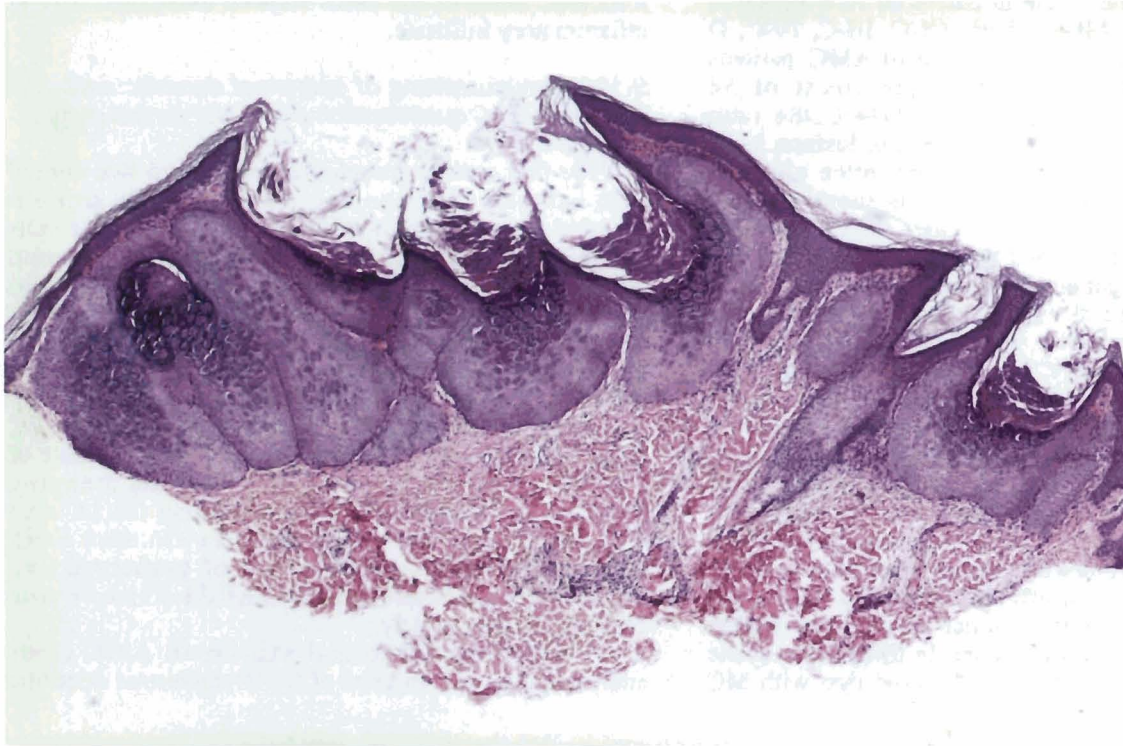


**Fig. 1.** Molluscum contagiosum lesions affecting eyelids in a patient suffering from AIDS.

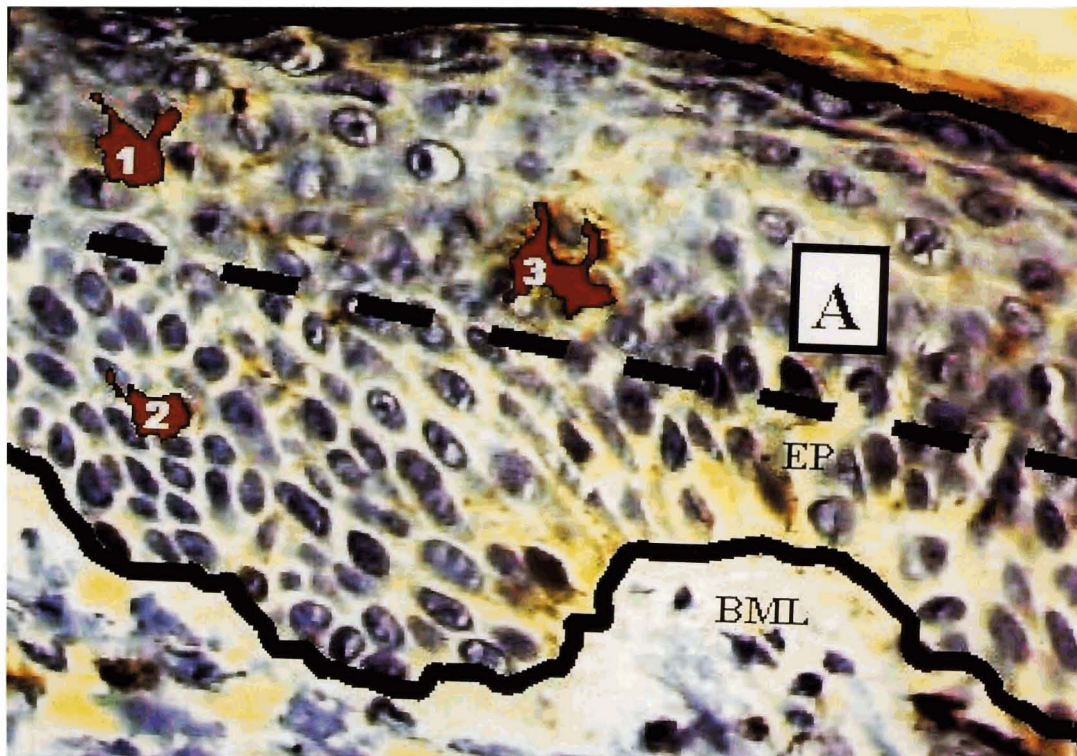
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cells within the lesional areas was noted, whereas in the perilesional epidermal skin there were some dendritic cells in a variable number ranging between 256.25 and

110.89 LC per  $\text{mm}^2$  of epidermis with a mean value of  $174.45 \pm 33.63$  LC/ $\text{mm}^2$ . In perilesional sites the LC area represented  $0.69 \pm 0.20$  % of the total epidermal area.



**Fig. 2.** Histological features of molluscum contagiosum of multilobular character affecting HIV+ patient HE, x40



**Fig. 3.** Screen displays of a microscopical captured image in computer morphometry system showing the analyzed parameters: epidermal length (EP), basement membrane length (BML), epidermal area (A) and surface area of LCs. Areas of three S-100-positive epidermal dendritic cells are enhanced in red (Frame area captured,  $0.030948$   $\text{mm}^2$ ).

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When considering epidermal and basement membrane lengths, the mean number of LC in perilesional sites was  $14.80 \pm 3.06$  and  $10.05 \pm 1.44$ , with values fluctuating between 4.61 and 24.61, and 3.92 and 12.72 respectively. In a direct microscopical observation, S-100-positive cells often appeared, gradually increasing when remoteness of the lesion was larger. Moreover, in the areas with high S-100-positive count, the LC had more prominent dendrites and were subjectively more intensely stained than in areas with low counts.

In the second control group made up of typical cases of MC, affecting three non-AIDS patients, we also observed a lack of LC at lesional level; however, the number of S-100-positive dendritic cells in perilesional epidermis was slightly higher, with a mean value of  $182.10 \pm 67.77$  LCs/mm<sup>2</sup> (ranging between 177.78 and 338.60 LCs per mm<sup>2</sup>). In relation to cellular surface, LC area represented  $1.06 \pm 0.42$  % of the total epidermal area and LC numbers were also somewhat higher when considering an epidermal and basement membrane length unit (mm) with mean values of  $17.07 \pm 10.25$  and  $12.61 \pm 3.04$  respectively. All these counts and areas of LC found morphometrically in our study are given in Table 1. When comparing all these counts and areas we observed a reduction in the LC number in AMC with respect to MC in non-AIDS patients, and also with respect to normal samples. However, we found a significant difference only when considering the number of LC/mm of basement membranes in AMC versus the first control group, corresponding to normal skin samples (Mann-Whitney test,  $p=0.0105$ ; Student's T test,  $p=0.018$ ). When considering LC number by mm<sup>2</sup> of epidermal area, we found an almost significant difference (Mann-Whitney test,  $p=0.0550$ ) upon comparing the same groups (AMC versus normal skin samples).

## Discussion

LC was described as an intra-epidermal dendritic cell by Paul Langerhans in 1868, and nowadays, over a century later, it is well-known that LCs play a vital role in the skin immune system as potent antigen-presenting cells constituting an important arm of the body's initial line of immunological defence (Breathnach, 1988). A

considerable body of evidence supports the view that altered LC-lymphocyte interactions may be central to the pathogenesis of certain skin diseases, including diverse dermatological conditions in HIV-infection. In this study we performed a morphometric count of the S-100-positive suprabasal epidermal cells in a series of AMC in HIV+ patients as well as in control groups of normal skin and MC in non-AIDS patients, in order to evaluate the possible alterations of LC populations that may explain the atypical growth of these lesions.

A prime initial aspect of interest was to assess the effectiveness of S-100 immunostaining as a marker of LC. Usually LC are identified by means of epidermal ATPase activity or by the demonstration of CD1a (OKT6), CD4, HLA-DR or other cell surface antigens (Breathnach, 1988). However, these procedures often require unfixed frozen tissue, not always available in routine diagnostic practice and above all in retrospective analyses, as in our study based on archive material. On the other hand, these markers are also frequently present in other immune cells, and thus in pathological skin may not be suitable markers for LCs. Neither is S-100 positivity specific for LCs because it is also present in epidermal basal melanocytes, despite which, counts of S-100-positive epidermal dendritic cells seem to reflect the total number of LCs demonstrated by other staining methods in frozen material (Prieto et al., 1998), and different studies have found its immunocytochemical demonstration to be useful and reproducible (Shaw and Fletcher, 1991), above all when employing a double stain with S-100 and L-Dopa (Halliday et al., 1986), or enumerating only the suprabasal cells (Shaw and Fletcher, 1991; Prieto et al., 1998). In this sense, to avoid epidermal melanocyte counts as LC, in our study we considered only the intraepidermal S-100 immunoreactive cytoplasmic cells located over the basal layer, which displayed a distinctive nucleus and at least one dendritic cytoplasmic process.

Another aspect was the methodological procedure to assess the LC network in epidermis, given that enumeration methods used can influence the quantification of LCs in normal and pathological skin (Bieber et al., 1988). Some authors indicate that for vertical sections, LCs should be measured with respect to epidermal length rather than epidermal area or

**Table 1.** Enumeration (number $\pm$ SD) of S-100 positive epidermal Langerhans cells (LC)<sup>a</sup>.

METHOD OF MEASUREMENT <sup>b</sup>	STUDY GROUP (No. of samples)		
	AMC in HIV+ patients <sup>c</sup> (7)	MC in non-AIDS patients <sup>c</sup> (3)	Normal skin samples (5)
LC/mm of epidermal length	14.80 $\pm$ 3.06	17.07 $\pm$ 10.25	21.79 $\pm$ 1.76
LC/mm of basement membrane length	10.05 $\pm$ 1.44*	12.61 $\pm$ 3.04	16.02 $\pm$ 1.36
LC/mm <sup>2</sup> of epidermal area	174.45 $\pm$ 33.63	182.10 $\pm$ 67.73	284.84 $\pm$ 36.95
LC area of LC with respect to epidermal area (%)	0.69 $\pm$ 0.20	1.06 $\pm$ 0.42	0.83 $\pm$ 0.10

<sup>a</sup>: LC were identified considering only S-100 positive intraepidermal dendritic cells of suprabasal location; <sup>b</sup>: in all measurement methods five HPF (0.030948 mm<sup>2</sup> of frame area) were counted using computer morphometry; <sup>c</sup>: in each sample five HPF in the perilesional epidermis were counted; \*: significantly less than in normal skin samples (Mann-Whitney test,  $p=0.0105$ ; t-student test,  $p=0.018$ ).

epidermal-dermal junction length, since the LCs form a single layer of interlinking cells which undulate throughout the epidermis with only one LC in a vertical plane (Halliday et al., 1986), so that the number of LCs in the epidermis appears to be independent of epidermal thickness. On the other hand, several authors have postulated that LC density may be inversely related to cell volume (Yu et al., 1994), above all in skin under ultraviolet light exposure. In many skin lesions epidermal thickness varies, and when counts of LC per unit area are performed, it could be that alterations in epidermal thickness influencing the result (Lisi, 1973) reveal lower counts in skin diseases with thickened epidermis and increased counts in skin diseases with reduced epidermal thickness. Thus we estimated the absolute and relative surface areas of LC with respect to epidermis area as a parameter of the epidermal surface occupied by LC, but we found no significant differences when considering the relative area of LC in the different study groups. Quantification of LC by enumeration per mm length of basement membrane, or counting LCs per 200 basal cells (Ashworth et al., 1986), can also be considered as a convenient method (Halliday et al., 1986), but in papillomatosis skin diseases with very convoluted basement membrane, results may prove less reliable (Bieber et al., 1988). All these facts show that the density of the LC pool is not identical in different skin diseases with acantosis and papillomatosis, and to avoid these inconveniences, we also performed a morphometric analysis using three different counts, referring to epidermal and basement membrane lengths as well as to epidermal area.

Our study has shown, by means of S-100 immunostaining, a complete lack of LC, in the lesional epidermis of MC affecting both HIV+ and healthy non-AIDS patients. This finding is in agreement with other reports that have also shown the disappearance of LCs in the molluscum lesional area, as well as in other viral skin lesions using S-100 protein and other cellular markers of LC (Drijkoningen et al., 1988; Viac and Chardonnet, 1990). This absence probably indicates a toxic effect of different viruses on dendritic cells. However, the host-virus relationships in MC are very peculiar and, in contrast to other poxviruses such as variola, MCV invokes scant cell-mediated response, and MC lesions begin relatively immunologically isolated (Heng et al., 1989; Gottlieb and Myskowski, 1994; Senkewich et al., 1996), devoid of immunocompetent cells including the absence of LC and T-lymphocytes within MC lesions (Heng et al., 1989; Viac and Chardonnet, 1990), all of which contrast with a normal or increased density of LC and T-cells in perilesional skin. This lack of immunocompetent cells within MC may be due, according to recent studies, to the production of a protein coded by an MCV gene that acts as a chemokine antagonist and inhibits inflammation (Senkevich et al., 1996; Damon et al., 1998).

In AMC affecting HIV+ patients we also found a decrease in the number of LCs in the perilesional

epidermis, and this reduction in the number of LCs is statistically significant when comparing AMC with respect to normal skin, but only when considering LC counts related to basement membrane length. These results imply two facts that are to be emphasized: firstly, that LC counts with respect to basement membrane length are probably the most reliable and effective parameter to evaluate and compare LC populations between normal and pathological skin of acanthotic type, such as MC; and secondly, that S-100 epidermal dendritic cell depletion in perilesional MC epidermis may be the result of a direct viral cytotoxic effect of HIV on epidermal LC. Lastly, the latter could explain the clinical challenge of MC in immunocompromised people.

MC is common in HIV patients and its prevalence ranges from 5% to 18%, although as many as one third of patients with CD4 counts of less than 100 cells/mm<sup>3</sup> may be infected (Izu et al., 1994), and at the same time giant molluscum lesions (> 1cm) have been reported as good markers for decreasing helper T-cell counts (Koopman et al., 1992). Thus, MC is an indicator of late-stage HIV infection (Schwartz and Myskowski, 1992), probably because of an important decrease of LC in the skin immune system. In this sense it is known that LCs bear CD4 receptors which make them potential targets for the HIV-1 virus (Stingl et al., 1990; Zambruno et al., 1991), and some studies point out that the skin LC numbers could be of prognostic value in HIV patients (Belsito et al., 1984) and have a good correlation with T4 cells counts (Dreno et al., 1988).

In spite of these data, involvement of LCs in HIV infection remains controversial. Although some authors have found an LC reduction in clinically normal skin of HIV-infected patients (Izu et al., 1994; Koopman et al., 1992), indicating a direct cytotoxic effect of the HIV virus, others have not found any decrease in their number (Becker et al., 1988; Kalter et al., 1991). Furthermore, recently Nandwani et al. (1996) found no correlation between CD4 cell counts and epidermal LC numbers in clinically normal skin of HIV+ patients, but other authors have shown that LC density decreases locally in different skin diseases in HIV+ patients (Valcuende-Cavero et al., 1988; Zimmelman et al., 1994). These differences are often difficult to explain due to different methodologies employed in LC quantification. Furthermore, these discrepancies may be a consequence of studying only static dendritic cells accessible to immunohistochemical examination. Future studies should be directed at measuring the number of epidermal LCs together with dynamic studies, to further clarify the functional integrity of the antigen presenting cells of epidermis, both resident as well as circulating (Gillian et al., 1998), in the atypical growth of MC in HIV+ patients.

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