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Quantitative analysis of T6-positive Langerhans cells in human skin cancers* **

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Summary. Epidermal Langerhans cells (LC) are required for antigen-presentation and for stimulating antigen-specific T cell activation. Similar functions may be important in the immune response to malignant skin tumours. Monoclonal anti-T6 antibody was used to examine LC population in basal and squamous cell carcinomas. Positive control labeling was performed with monoclonal anti-HLA-DR antibody.

The number of T6-positive LC per mm^2 of section was significantly decreased ($p<0.01$) in the tumour group in comparison with a sex and age-matched control group. The number of sun-exposed and covered regions was taken into consideration in each respective group. Within the tumours, LC were found more frequently in the tumour periphery and in most differentiated tumour areas (horn pearls) than in the rest of the tumour mass. T6-positive LC were rarely found in the dermis. Moreover, LC exhibited morphological changes in specimens from tumours. Staining with anti-HLA-DR antibody revealed less numerous positive cells within tumour nests than labeling with OKT6. A relationship between T6-positive LC quantities and extent of HLA-DR-positive infiltrates around tumours could not be established. These results suggest that immunological surveillance of neoantigen-bearing tumour cells may be impaired in skin cancer. A reason for the reduced LC number may be an altered microenvironment in tumour tissue.

Key words: Langerhans cells – Basal cell carcinomas – Squamous cell carcinomas – Quantitative analysis

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Introduction

Langerhans cells (LC) make up 3–5% of the epidermal cell population and form a reticuloepithelial trap for allergens (Shelley and Juhlin 1976). They bear Fc and C3 receptors (Stingl et al. 1977), express certain membrane antigens like HLA-DR (Rowden et al. 1977) and T6 (Murphy et al. 1981) and membrane-bound ATPase (Wolff and Winkelmann 1967). They are required as antigen-presenting cells (Silberberg et al. 1976; Stingl et al. 1978b) and for the stimulation of antigen-specific T cell activation (Stingl et al. 1978a; Braathen and Thorsby 1980).

Similar functions may be important in the host immune response to malignant epithelial skin tumours. These tumours are known to be induced by chronic exposure to ultraviolet light (UV) and to occur most frequently in sun-exposed regions of elderly persons (Epstein 1983). Chronic UV exposure clearly diminishes the number of LC (Zelickson and Mottaz 1970; Gilchrest et al. 1983). Aging is another factor reducing LC numbers (Gilchrest et al. 1982; Thiers et al. 1984). The development and growth of epithelial skin tumours may therefore be favored by the impaired immunological surveillance of neoantigen-expressing tumour cells resulting from reduction of LC number and function. In the present study, we performed a quantitative analysis of LC population in basal (BCC) and squamous (SCC) skin carcinomas in comparison with normal epidermis of a sex and age-matched control group, taking into consideration the number of sun-exposed and covered regions in each group.

Materials and methods

Subjects

17 tumours were obtained from 8 male and 9 female patients, aged between 58–101 years (mean 75 years). 13 tumours were from sun-exposed and 4 from covered skin regions (Table 1).

Table 1. T6-positive LC quantitation in malignant epidermal tumours

Patient N°	Age	Sex	Localization	Histopathological diagnosis		T6-positive cells per 1 mm^2 of tumor section	Intensity of HLA-DR-poisitve peritumoral infiltrate
1	94	W	chin	adenoid	BCC	3.6	+ + + +
2	73	W	cheek	keratotic	BCC	19.7	+ +
3	83	M	nose	keratotic	BCC	16.8	+
4	68	W	nose	keratotic	BCC	25.3	+
5	78	M	nose	solid	BCC	36.6	+ + + +
6	85	W	nose	solid	BCC	0.7	+ + +
7	59	M	nose	solid	BCC	17.6	+
8	80	M	nose	solid	BCC	54.1	+ + +
9	75	W	forehead	solid	BCC	3.2	+ +
10	59	M	lateral neck	solid	BCC	4.4	+ + +
11	76	W	shoulder	superficial	BCC	3.7	+ + + +
12	67	M	back	superficial	BCC	34.6	+ + +
13	70	W	upper lip		SCC	16.8	+ + +
14	64	M	ear		SCC	29.4	+ + + +
15	101	W	forehead		SCC	9.2	+ +
16	58	M	lower leg		SCC	11.7	+ +
17	79	W	lower leg		SCC	8.7	+ + +

BCC = basal cell carcinoma; SCC = spinal cell carcinoma; M = man; W = woman.
(+) little; (+ +) moderate; (+ + +) strong; (+ + + +) very intense

The control group consisted of 12 healthy volunteers (6 female, 6 male) aged between 56–92 years (mean 72 years); 9 biopsies were taken from sun-exposed (back of the hand) and 3 from covered skin regions (inner upper arm).

Histology techniques

Tumours were excised under local anaesthesia with 2% lidocaine. The samples were divided, one part being processed for routine paraffin histology. Other parts of the tissue were snap-frozen in liquid nitrogen and stored at −70° C.

4 µm-thick cryocut serial sections were air dried at room temperature, fixed in acetone (10 min. at −20° C) and incubated wtih 1:10 diluted (PBS, pH 7.4) OKT6 (Ortho Immunbiology, Raritan, N.J., USA) monoclonal antibody (MCA) or with 1:50 diluted (PBS, pH 7.4) anti-HLA-DR MCA – JOT2b (Immunotech, Marseille, France) for 30 min at 37° C. After having been rinsed in PBS with 1% BSA (2 × 15 min), sections were incubated with 1:30 diluted (PBS, pH 7.4) FITC-conjugated goat anti-mouse mIg (IgA/IgM/IgG) antiserum (Nordic, Tilburg, Netherlands) for 45 min at 37° C. After final washings in PBS, sections were mounted in buffered glycerine. Control slides were always prepared for each antibody layer by replacing the respective immune serum with PBS or with mouse immunoglobulins of the given IgG-type. Two of the serial sections were always stained with haematoxylin and eosin for control purposes.

Quantification

The T6-positive LC were counted in adjacent, non-overlapping high power fields (× 320) under an epifluorescent Polyvar microscope (Reichert-Jung, Vienna, Austria) fitted with an integrating eyepiece Zeiss II (Zeiss, Oberkochen, FRG). Only immunofluorescence (IF) positive cell bodies were counted, dendrites were left out.

The measurements of surfaces of the tissue sections were carried out with a semiautomatic image analyser Videoplan (Kontron, Munich, FRG). The end sum of measured surfaces for each biopsy was calculated according to the selected program. In each case, all the surface of lesional tissue or healthy epidermis (control group) present in the respective sections was measured; always at least 4 mm^2 on two representative slides. The horny layer and the keratotic centers of horn pearls were left out of the surface measurements. LC density was calculated as LC number per 1 mm^2 of the secions studied. The surfaces of specimens were measured on the same section fields used for LC counting, but with white light. The peritumoral HLA-DR-positive cellular infiltrates were semiquantitavely estimated: (+) little, (+ +) moderate, (+ + +) strong, and (+ + + +) very intense.

Statistical analysis

Statistical analysis of paired values was performed using Wilcoxon's T-test (Dagnelie 1975) with $p < 0{,}01$ indicating significant differences. For comparing T6-positive LC number in tumours with those of normal epidermis, the mean number of T6-positive LC per 1 mm^2 of epidermis section in 12 normal skin biopsies was paired with the value of each tumour.

The statistical comparison between mean values of each chosen pair of groups (e.g.: LC densities in tumours from sun-exposed regions and from non-exposed ones; LC densities in BCC and SCC) was performed with Mann-Whitney's *U*-test with $p < 0.01$ indicating significant differences.

Results

Clinical data and routine histological diagnoses are summarised in Table 1. Histological classification of basal cell carcinomas was based on the predominant differentiation type of each tumour. SCC always corresponded to grade I or II of SCC grading (Lever and Schaumburg-Lever 1983). In three cases of SCC, atrophic solar keratoses were partly

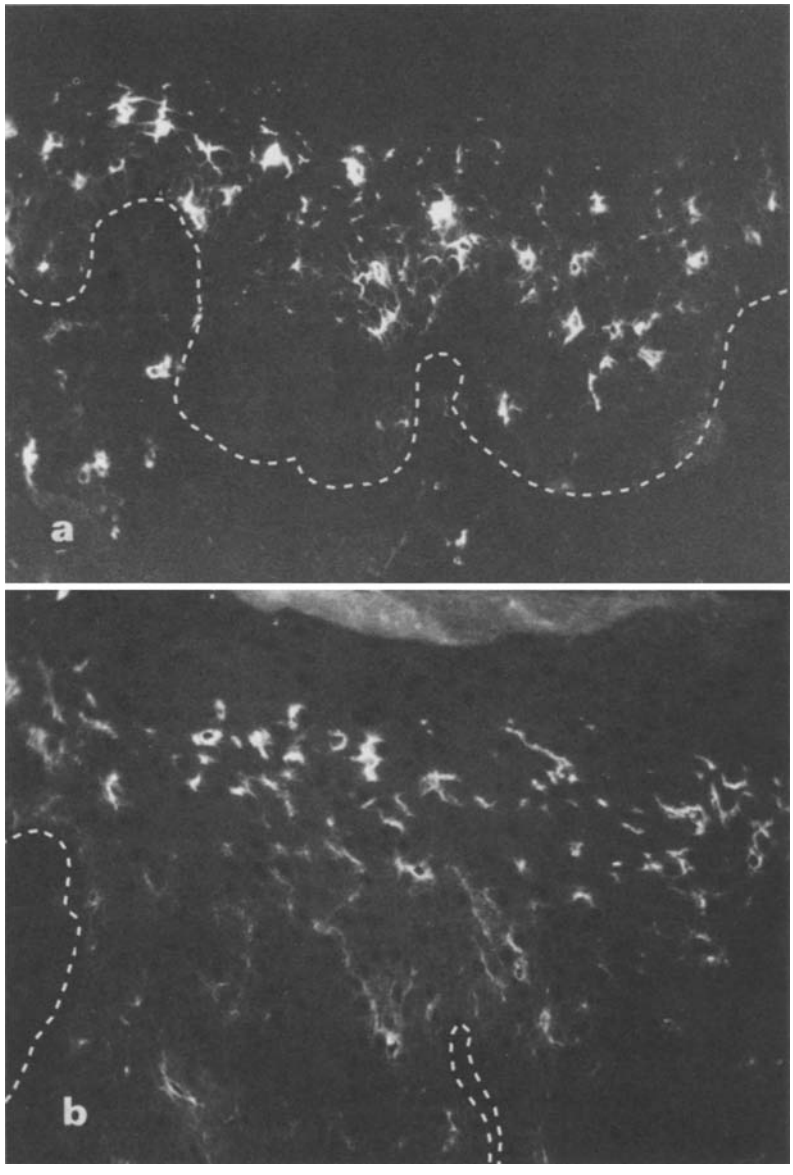

Fig. 1 a, b. Indirect immunofluorescence with OKT6 MCA:
a basal-cell carcinoma,
b spinal cell carcinoma.
Dermo-epidermal junction is marked with a broken line. Lower parts of epidermal compartment represent tumour proliferation controlled on haematoxilin/eosin-stained sections. Evident progressive reduction in T6 positive cells can be observed. ×250

included in peri-lesional epidermis; they were not included in calculation.

Control slides which had been prepared by omitting respective primary immune serums, were always negative. T6-positive LC were regularly distributed in normal epidermis (control group) from sun-exposed as well as from non-exposed areas. The mean number of epidermal T6-positive LC per mm^2 of normal epidermis section is given in Table 2. There are obvious differences in LC density between epidermis from sun-exposed and from covered areas.

The distribution of T6-positive LC in tumours, both in SCC and in BCC, was very irregular (Table 1, Fig. 1a, b). A gradual decrease of the LC number could be observed from overlying epidermis toward the center of the tumour nests. Generally, LC could be found more frequently in the tumour periphery and, in SCC tissue, around horn pearls (Fig. 2). In both types of tumours, T6-positive LC exhibited less and shorter dendrites than in epidermis from control group.

The mean number of T6-positive LC per 1 mm^2 of tumour section in each biopsy is given in Table 1. T6-positive LC were significantly diminished in tumours in comparison with LC in epidermis from control group ($p < 0.01$), irrespective of whether they were compared with sun-exposed or non-exposed epidermis (Table 2). No significant differences were found in the number of T6-positive LC between BCC and SCC. Nor was there a significant difference in the T6-positive LC

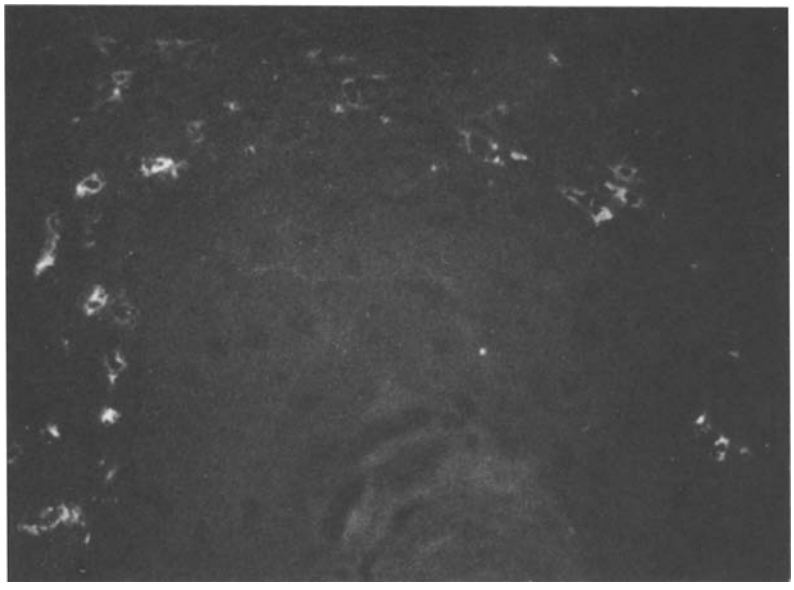

Fig. 2. OKT6-positive cells surrounding a horn pearl in spinal cell carcinoma. × 250

Table 2. Mean T6-positive LC number in normal epidermis and in skin carcinomas

Tissue	n	$\bar{x}$ (LC/mm^2)	±SD	Statistical analysis
Normal epidermis (total)	12	160.6	41.8	
from sun-exposed areas	9	143.4	24.4	S
from non-exposed areas	3	212.3	43.5	($p<0.01$)[a]
Skin carcinomas (BCC+SCC)	17	17.4	14.6	
BCC	12	18.3	16.8	NS
SCC	5	15.2	8.6	($p>0.01$)[b]
from sun exposed areas	13	18.2	15.4	NS
from non-exposed areas	4	14.7	14.7	($p>0.01$)[b]

n = number of biopsies; $\bar{x}$ = mean number; SD = standard deviation; S = significant difference; NS = not significant difference

[a] Wilcoxon's T-test; [b] Mann-Whitney's U-test

number between tumours deriving from sun-exposed and from covered regions. In dermal infiltrates around the tumour nests, T6-positive LC could only be occasionally seen (Fig. 1).

Immunofluorescence (IF) staining of tumour masses with anti-HLA-DR MCA (JOT2b) revealed less IF-positive cells than labeling with OKT6 MCA in general with the exception of case 8, where HLA-DR-positive cells slightly exceeded T6-positive ones. Labeling with anti-HLA-DR MCA confirmed the paucity of epidermal LC in tumours and suggested a scarcity of reactive cellular infiltrates inside tumour tissue (Fig. 3a, b). Within peritumourous stroma, HLA-DR-positive cellular infiltrates of variable extent could be observed. The coarse semiquantitative estimation of the infiltrates did not reveal any correlation between their intensity and LC density in tumour nests (Table 1).

Discussion

In a recent study on human epidermis, no statistically significant differencies have been found between regional mean densities of T6 antigen-bearing LC (per 1 mm^2 of section) apart from LC density in the soles of the feet (Berman et al. 1983). However, it has been shown that aging and chronic sun exposure may significantly decrease epidermal LC numbers (Zelickson and Mottaz 1970; Gilchrest et al. 1982; DeLeo et al. 1982; Thiers et al. 1984). Therefore, epidermis from the back of the hand (as sun-exposed region) and from the inner upper arm (as sun-protected region) were used to establish a matched control group in our study.

Our data present clear cut evidence that in human BCC and SCC the number of T6-positive LC is significantly diminished ($p<0.01$), independently of the site of tumour origin, in comparison with normal epidermis. There were no differences in LC density between BCC and SCC. In contrast to our findings, Gatter et al. (1984a) found the number of T6-positive LC in SCC similar to that in normal skin and a decreased LC number in BCC. This discrepancy is probably due to the "semiquantitative method" applied by those authors: the IF-positive cell densities were only estimated as "increased" or "reduced" without any objective measurements undertaken. Moreover, their study lacked a well-defined control group.

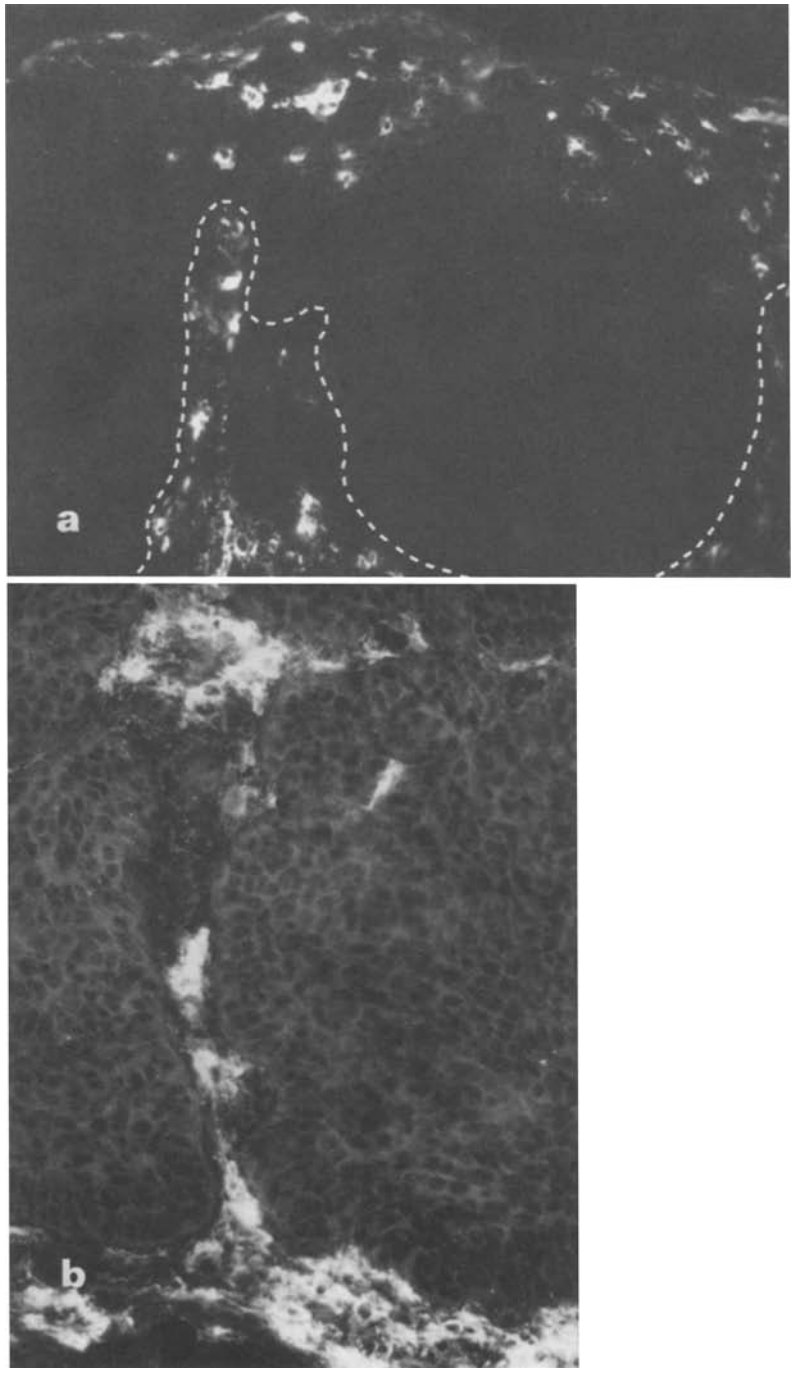

Fig. 3a, b. Anti HLA-DR staining of a basal cell carcinoma.
a Dermo-epidermal junction is marked with a broken line. Lower epidermal compartment lacking IF-positive cells represents tumour tissue. Sparse cellular dermal infiltrate is labelled. ×250.
b Higher magnification of another biopsy (×500). Stronger dermal infiltrate between tumour nests. One HLA-DR-positive cell can be observed within otherwise negative tumour tissue

Using the ATPase technique, LC were found in slightly increased numbers in BCC and in decreased numbers in SCC (Lisi 1973). However, ATPase activity has been demonstrated to be more sensitive to ultraviolet light than T6 and HLA-DR antigens (Tjernlund and Juhlin 1982; Czernielewski et al. 1984) and in absence of an appropriately matched control group one has to interpretate with caution the results obtained with this method in tumours.

It is noteworthy that we could not find statistically significant differences in the LC number between tumours derived from sun-exposed and from covered regions. In already established, infiltrating skin carcinomas, there does not seem to be any influence of UV-light on the density of T6-positive LC in tumour tissue. This finding may be explained in part by the depth of skin penetration by UV-rays: almost all the tumour tissue is out of range of UV radiation.

The number of HLA-DR-positive cells within tumour nests appeared to be always even lower than that of T6-positive cells (with the exception of the case 8). This may additionally reflect the scarcity of infiltrating HLA-DR-positive cells within tumour tissue.

Mononuclear cell infiltrations in skin carcinomas have been found to consist predominantly of T-cells (Gatter et al. 1984b; Guillén et al. 1985). These cells were associated with LC in a manner akin to that seen in delayed hypersensitivity reactions (Claudy et al. 1976; Murphy et al. 1983). In semithin sections, LC have been observed in close apposition to UV-damaged keratinocytes (Gilchrest et al. 1982) and, ultrastructurally, to neoplastic keratinocytes in BCC (Macadam 1978). Considering the antigen presenting capacity of LC (Stingl et al. 1978a; Bjercke et al. 1984) and their role in generating cytotoxic T-cells (Tsushida et al. 1983; Faure et al. 1984) it is reasonable to hypothesize that LC play a similar role in presenting neoantigens of malignant cells to T-lymphocytes, so initiating the cellular immune response to skin cancer. The role of LC in the host immune response to skin cancer remains questionable, however, with the T6-positive LC density highly diminished in BCC and SCC and with only rare LC in the peritumerous infiltrate. Additionally, no relationship between LC numbers within tumour nests and the intensity of the peritumoral infiltrates could be assessed.

The reasons for the significant reduction of T6-positive cells in skin carcinomas are not evident. The altered microenvironment could, at least in part, be responsible for the reduction of LC density. Neoantigen-expressing malignant cells, altered functional properties, and disturbed epithelial differentiation (Gatter et al. 1984b; Karienemi et al. 1984) would influence homing conditions for epithelial LC. In agreement with this is our observation that LC are often found most frequently in the periphery of tumours and around horn pearls of SCC, in keratinocyte differentiation areas.

In summary, we report on the greatly diminished number of T6-positive LC in human BCC and SCC. This may indicate an impairment of the immunological surveillance of neoplastic cells. The exact role of the LC in the host immune response to skin cancer is yet to be defined.

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