Quantitative Alterations in Cutaneous Langerhans Cells During the Evolution of Malignant Melanoma of the Skin

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Melanomas are associated with a T-cell predominant infiltrate that may cause their regression. Langerhans cells (LC) are essential for initiation and maintenance of specific T-cell-mediated responses in the skin. Therefore, a change in this antigen-presenting LC population may alter the host response. To determine whether the LC population varies during the evolution of primary cutaneous melanoma 32 melanocytic lesions, nevi, and cutaneous melanomas were studied by quantitative immunohistology. The monoclonal antibody, Leu-6, and the avidin biotin complex immunoperoxidase method were used to identify LC. Compared with histologically normal melanoma-adjacent skin, epidermal LC were depleted above "deeply invasive" melanomas but were relatively unchanged above nevi, "early invasive" melanomas, and cutaneous metastatic melanoma nodules. Dermal LC were significantly increased around in situ and "early invasive" melanomas but not around "deeply invasive" melanomas or cutaneous metastatic nodules. Dermal LC are thus associated with early transformed melanocytes and may present neoantigens to T lymphocytes in situ or after LC maturation in the draining lymph node. Melanoma-associated LC decline in number as melanoma progresses. *J Invest Dermatol 91:125–128, 1988*

Regression of primary cutaneous melanomas (CM) is associated with dermal lymphocytic infiltrates [1–2] comprised predominantly of T lymphocytes (T cells), a few B lymphocytes (B cells), macrophages, and Langerhans cells (LC) [3–6]. In normal epidermis, LC comprise 2%–6% of the cells, but they form a network that facilitates their interaction with antigens entering the skin [7]. Such antigen-pulsed LC are efficient antigen-presenting cells (APC) that are essential for initiation and maintenance of cutaneous immune responses [7]. Any change in the LC population may regulate host resistance and thereby favor either the progression or regression of melanoma. To determine if the LC population varies with the evolution of melanoma, we systematically quantified LC associated with paired samples of skin adjacent to and containing a wide range of melanocytic tumors.

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Abbreviations:

APC: antigen-presenting cell
CM: cutaneous melanoma
DLC: dermal Langerhans cell
DTH: delayed type hypersensitivity
ELC: epidermal Langerhans cell
LC: Langerhans cell
S-100: S-100 protein

MATERIALS AND METHODS

Immunostaining Tissue slices (<10 mm by 4 mm) for Leu-6 immunoperoxidase staining were removed from unfixed surgical specimens within 1 h of surgery (Table I). Slices included 50% of clinically normal, lesion-adjacent skin and 50% of lesion-containing skin. To avoid artifacts, slices were removed from the center of the specimen and areas of necrosis or scarring were not sampled. Slices were immediately embedded in O.C.T.™ (Lab-Tek Division, Miles Laboratories, Naperville, IL), snap frozen in an isopentane/dry ice bath, and stored at −70°C until cryostat sectioning. These blocks, stored for less than 1 wk, were vertically sectioned (5μm, −20°C), dermal edge first, placed on acetone-cleaned microscope slides, fixed in dry acetone (4°C) for 5 min, and air-dried for 2 h. Sections from all cases were incubated with Leu-6 [8,9] (1–4μg/ml) and case 1960.1 was also incubated with Leu-HLA-DR [10] (0.07-0.1μg/ml) (Becton Dickinson, Mountain View, CA). All sections were then incubated with biotinylated horse anti-mouse IgG and finally peroxidase conjugated biotin-avidin complexes (Vector Laboratories, Burlingame, CA) [11].

For anti-S-100 protein (S-100) staining of LC [12] archived paraffin blocks were selected from case 1960.1 to provide 50% lesion and 50% normal adjacent skin. To avoid confusion between intraepidermal S-100 positive melanoma cells and ELC a nodular melanoma not involving the suprabasal epidermis was studied. Normal melanocytes were distinguished from LC by their basal location and relatively weak staining with anti-S-100 protein. These paraffin-embedded tissues were processed as described previously [13]. They were incubated with a polyclonal rabbit anti-bovine S-100 protein antiserum [13] (1:50–1:200 dilution), followed by swine anti-rabbit immunoglobulin, and finally rabbit anti-peroxidase-peroxidase complexes (Dako, Inc. Santa Barbara, CA).

Transmission Electron Microscopy Additional tumor and normal adjacent tissues from case 8070.1 were fixed for LC identifica-

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tion by electron microscopy [14]. Ultrathin sections for electron microscopy were stained with uranyl acetate and lead citrate. Electronmicrographs were prepared on a Zeiss EM 109 microscope and analyzed.

Image analysis All immunohistology slides were analyzed with an IBM-PC-assisted image analysis system (Southern Microcomp Instruments, Atlanta, GA). At 40X every 300μm width of skin was further magnified 10X, detected by a Dage B/W video camera, displayed on a Sony monitor, and evaluated for positively stained cell bodies for Leu-6, Leu-HLA-DR, and S100. The image analyzer was concomitantly used to accurately ($\pm$3%) and precisely ($\pm$ 1.7%) compute epidermal area for each 300 μm width of skin. Data are reported separately as epidermal LC (ELC) count (ELC/300μm), epidermal LC density (ELC/mm^2 epidermis), and as dermal LC (DLC) count (DLC/300μm). Stratum corneum and melanocytic nests do not contain LC and were not included in measurements of epidermal area. Adnexa-related epithelium, which generally has more LC than surface epidermis, was excluded. All fields on serial H and E stained slides were assigned a pathological designation [2] without prior knowledge of the immunohistological findings. In some slides more than one pathological change existed (Table I).

Data analysis Comparisons between data from tumor containing and normal fields within an individual case (mean $\pm$ S.D.) or between groups of similarly designated lesions (mean $\pm$ S.E.M.) were evaluated by the Wilcoxon Rank Sum test and the paired t test, respectively.

Table I. Description of specimens

Specimen	Anatomic Site	Pathology Diagnosis	Clark Level	Antibody
8352.1	Shoulder	CN*		LEU-6
2951.1	Back	CN		LEU-6
3838.1	Shoulder	IDN		LEU-6
8097.1	Calf	IDN		LEU-6
780.1	Back	IDN		LEU-6
1084.2	Calf	IDN		LEU-6
3303.1	Neck	IDN		LEU-6
3303.2	Neck	IDN		LEU-6
9777.1	Calf	DN		LEU-6
8352.1	Shoulder	DN		LEU-6
5429.1	Back	DN		LEU-6
5429.2	Leg	DN		LEU-6
9037.1	Back	LMIS	I	LEU-6
7423.1	Scalp	LMIS	I	LEU-6
8728.2	Ankle	LMIS	I	LEU-6
8070.1	Scalp	LMIS	I	LEU-6
80.1	Arm	SIS	I	LEU-6
477.1	Flank	SIS	I	LEU-6
7901.2	Back	SIS	I	LEU-6
6386.1	Calf	SIS	I	LEU-6
9480.2	Calf	SIS	I	LEU-6
8728.1	Ankle	LMM	II	LEU-6
8070.1	Scalp	LMM	II	LEU-6
8852.1	Vulva	MLM	II	LEU-6
1084.1	Calf	SSM	II	LEU-6
3303.1	Neck	SSM	II	LEU-6
4804.2	Back	SSM	III	LEU-6
7070.1	Back	SSM	II	LEU-6
7901.2	Back	SSM	II	LEU-6
7901.1	Back	SSM	III	LEU-6
8692.1	Scalp	LMM	IV	LEU-6
1960.1	Buttock	NM	V	LEU-6
				LEU-HLA-DR
1960.1	Buttock	NM	V	S100
9480.1	Calf	SSM	IV	LEU-6
9480.2	Calf	SSM	IV	LEU-6
9945.1	Knee	MCM		LEU-6
2890.2	Arm	MCM		LEU-6
1347.1	Breast	MCM		LEU-6

CN: Compound nevus; DN: Dysplastic nevus; IDN: Intradermal nevus; LMIS: Lentigo maligna melanoma in situ; LMM: Lentigo maligna melanoma; MCM: Metastatic cutaneous melanoma; MLM: Mucosal lentigenous melanoma; NM: Nodular melanoma; SIS: Superficial spreading melanoma in situ; SSM: Superficial spreading melanoma

RESULTS

To evaluate LC population changes, during the evolution of CM, samples were divided into 5 groups; dysplastic nevi, melanoma in situ (Clark level I), "early invasive" CM (Clark levels II, III), "deeply invasive" CM (Clark levels IV, V), and cutaneous metastatic melanoma. For comparison, non-dysplastic melanocytic nevi were also studied.

Epidermal Langerhans Cells (Table II)

Nevi, melanoma in situ, and "early invasive" melanomas: The LC distribution above these groups of melanocytic lesions was similar to that of the normal adjacent epidermis. ELC were insignificantly reduced but uniformly distributed over 3 of 9 in situ CM and 6 of the 8 "early invasive" CM. In some of these cases, small areas of epidermis were devoid of LC. These regions were typically over dense dermal foci of LC-containing lymphocytic infiltrates.

"Deeply invasive" melanomas: A significant reduction was observed in both the number and density of the epidermal Leu-6 population overlying the 4 "deeply invasive" CM. All histogenetic types were depleted of ELC; the smallest reduction was seen over the Lentigo maligna melanoma (data not shown).

Figure 1 shows the abrupt depletion of Leu-6$^+$ cells (3.2 $\pm$ 3.2/300μm epidermis, 19 fields, v. 10.9 $\pm$ 4.9, 26 fields $p = 0.0001$) in the buttock epidermis above a deeply invasive nodular CM (Case 1960.1).

Mechanism of ELC reduction: Several common causes of LC depletion have been previously defined and were evaluated in our study [15–17]. Acanthosis can cause an apparent reduction in ELC. In this study, "deeply invasive" CM had a statistically insignificant acanthosis (epidermal thickness: .041 $\pm$ .0081 mm^2/300μm v .027 $\pm$ .0035, $p = 0.16$) and ELC reduction occurred in thinned epidermis as well as acanthotic epidermis overlying these "deeply invasive" CM. ELC may be depleted by mechanisms involving ultraviolet light [17]. We found ELC were depleted over deeply invasive melanomas from sun exposed and sun protected sites (data not shown). Modulation of a single cell surface marker could also cause an apparent depletion of ELC. To determine whether ELC depletion was due to cell loss or reduced expression of the Leu-6 epitope (CD1), we studied Case 1960.1 for the expression of another ELC surface marker, HLA-DR and a *cytoplasmic* LC marker, S-100 protein, and

Table II. Epidermal Leu-6$^+$ cells are depleted above "deeply invasive" melanomas

Group	LC count (ELC/300 μm)		LC density (ELC/mm^2)	
(n)	Normal[a]	Tumor[b]	Normal[a]	Tumor[b]
Benign Nevi (7)	6.4 $\pm$ .78[c]	5.3 $\pm$ .71	394 $\pm$ 53	361 $\pm$ 45
Dysplastic Nevi (4)	4.4 $\pm$ .76	3.8 $\pm$ .34	301 $\pm$ 40	247 $\pm$ 29[d]
In Situ Melanoma (9)	4.8 $\pm$ .54	5.6 $\pm$ 1.0	350 $\pm$ 31	296 $\pm$ 53
Early Invasive Melanoma (9)	5.2 $\pm$ .84	5.4 $\pm$ 1.2	362 $\pm$ 53	230 $\pm$ 56
Deeply Invasive Melanoma (4)	8.0 $\pm$ 1.3	2.8 $\pm$ .64[d]	317 $\pm$ 46	83 $\pm$ 37[d]
Cutaneous Metastasis (3)	5.8 $\pm$ 2.2	6.9 $\pm$ 2.8	259 $\pm$ 53	285 $\pm$ 77

[a] Microscopically normal epidermis adjacent to tumor.
[b] Epidermis overlying tumor.
[c] All data mean $\pm$ s.e.m.
[d] $p < 0.05$

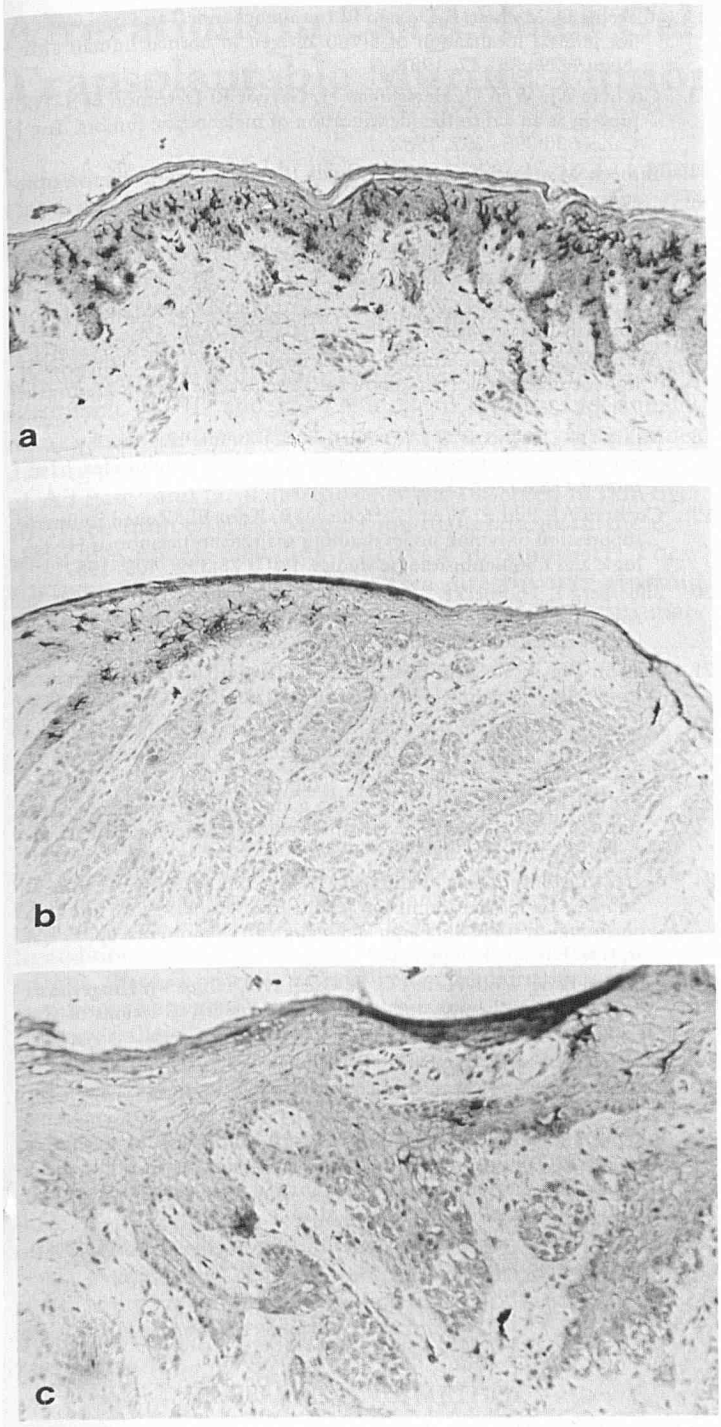

Figure 1. *a*, epidermal Leu-6$^+$ cells are numerous and uniformly distributed in skin adjacent to a deeply invasive nodular melanoma, 1960.1 (X90). *b*, epidermal Leu-6$^+$ cells are markedly depleted over this deeply invasive nodular melanoma (X90). Leu-6$^+$ cells which remain above the melanoma are confined to the edge of the tumor and have small abnormal dendrites. *c*, leu-6$^+$ cells are also depleted in the acanthotic epidermis above the tumor. Many small unstained lymphocytes, but few Leu-6$^+$ cells, are present in the tumor containing dermis (X225).

found a similar reduction of ELC stained by these markers (Leu-HLA-DR; 2.1 ± 2.0/300μm epidermis, 24 fields v. 7.4 ± 4.2, 17 fields, p = 0.0001 and S100 5.8 ± 4.1/300μm epidermis, 37 fields v. 11.4 ± 3.7, 11 fields, p = 0.0012). ELC depletion was predominantly, but not strictly, limited to only "deeply invasive"" melanomas. One "early invasive" LMM (Case 8070.1, Clark level II) had a significant reduction in ELC count and density (1.6 ± .79 Leu-6/300μm, 7 fields v. 3.2 ± 1.6, 6 fields, p < 0.04 and 51 ± 21 Leu-6/mm^2 v. 294 ± 126, p < 0.0004). This ELC depletion was qualitatively confirmed by transmission electron microscopy.

Dermal Langerhans Cells (Table III)

Nevi, melanoma in situ, and "early invasive" melanomas: Only occasional Leu-6$^+$ cells were observed in the normal dermis adjacent to pigmented lesions (1.2 ± 0.14/300μm, n = 31). Benign nevi, in situ CM, and "early invasive" CM all showed a significant increase in dermal Leu-6$^+$ cells. A statistically insignificant increase in dermal Leu-6$^+$ cells was observed in dysplastic nevi.

"Deeply invasive" melanomas: A statistically insignificant increase in dermal Leu-6$^+$ cells was observed around "deeply invasive" melanomas.

DISCUSSION

The epidermis over "deeply invasive" melanomas was significantly depleted of LC. The cause of this depletion is unclear but is not due to the loss or modulation of LC markers, ultraviolet light [17], or pathological changes, such as necrosis, regression, and scarring, that are known to cause LC depletion (unpublished observation and Refs 15 and 16). More novel mechanisms may be involved in the ELC depletion. For example, tumor-derived substances are known to affect a number of lymphocyte functions [18,19]. A diffusible melanoma-derived factor might adversely affect LC migration, differentiation, and function in the skin. However, LC were normal or increased in number and distribution in the epidermis immediately adjacent to primary melanomas (Fig 1). The stage of the immune response may also affect the presence of LC. LC involved in the development of immune responses may traffic more readily from the epidermis to regional lymph nodes [20]. Once established, the presence of the dermal lymphocyte infiltrate could alter the normal migration or differentiation of LC precursors. LC precursors may be preferentially recruited to the melanoma-associated dermal lymphocytic infiltrate by infiltrate-derived cytokines. Such a mechanism would eventually short-circuit the usual migration pathway to the epidermis [21]. Lastly, infiltrate-induced enlargement of the epidermis [22–23] may accelerate turnover of all epidermal cells including existing LC [23]. In total these changes may lead to a gradual reduction in ELC.

Other investigators [24,25] have reported cases with fewer ELC above "deeply invasive" malignant melanoma while others [26–27] have reported no change or an increase in LC above melanomas. To our knowledge, our study is the first to fully control and quantitatively analyze LC associated with melanomas.

Leu-6$^+$ cells were increased in the dermis deep to melanoma precursors and significantly increased below in situ and "early invasive" melanomas. "Deeply invasive" melanomas were *not* asso-

Table III. Dermal Leu-6$^+$ cells are increased in early melanomas.

Group	n	Dermal LC count (LC/300μm) Normal	Tumor
Benign Nevi	7	1.6 ± .23[a]	2.2 ± .28[b]
Dysplastic Nevi	4	0.8 ± .34	3.1 ± 1.2
In Situ Melanoma	9	1.3 ± .38	4.5 ± 1.0[c]
Early Invasive Melanoma	9	0.8 ± .19	3.8 ± .57[c]
Deeply Invasive Melanoma	4	1.2 ± .33	2.2 ± .92
Cutaneous Metastasis	3	0.9 ± .48	0.8 ± .35

[a] All data mean ± s.e.m.
[b] $p<0.05$.
[c] $p<0.01$.

ciated with *significantly* more dermal LC. The T-cell-predominant infiltrate associated with invasive melanomas is also diminished as melanoma progresses [4,5,27]. The reduction in the T-cell infiltrate is caused by immunoregulatory mechanisms that limit the DTH response [28]. LC are the focal APC in these responses and their reduction may therefore contribute to a diminished T-cell response.

In summary, LC and T cells are associated with early melanomas. Melanoma-associated antigens, in the early stages of melanoma development, would therefore appear to be presented by LC. The decline in the LC population may partially diminish the lymphoid infiltrate and its anti-tumor effect. Methods which counteract this decline or increase and sustain LC may result in more successful immunologically-mediated regression of primary melanoma.

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