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Immunological and histochemical analysis of regional variations of epidermal Langerhans cells in normal human skin

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Summary

Epidermal Langerhans' cells (LC) were enumerated in normal human skin from various anatomical sites using a monoclonal antibody (NA1/34) to human thymocyte antigen (HTA-1) and the standard ATPase reaction on frozen sections. The same population of cells was identified with each technique. LC densities were found to be significantly higher in hair bearing skin than in skin from the palm and sole. LC were also identified in hair follicles (where the numbers decreased from the superficial to the deep portions) and sebaceous glands but in no other adnexal structure. Normal numbers were encountered in patients who had received radiotherapy or systemic chemotherapy for malignant disease for periods of greater than two months before death. As LC are important antigen presenting cells, the variation in their density suggests that the immunological properties of normal skin may not be uniform throughout the body. This may be related to the varying anatomical distribution of some skin disorders with an immunological basis.

Introduction

Langerhans' cells (LC) are a consistent feature of the epidermis as well as in a variety of other squamous epithelial sites (Hutchens *et al.*, 1971; Al Yassin & Toner, 1976), the thymus and peripheral lymphoid tissues (Rausch *et al.*, 1977). Functionally, LC are important in initiating the allergic immune response by trapping external allergens (Shelley & Juhlin, 1976) and transporting antigen to effector T cells in regional lymph nodes (Silberberg-Sinakin *et al.*, 1976). The migratory properties of these cells has been clearly demonstrated in experimental contact hypersensitivity reactions (Silberberg *et al.*, 1976; Gschnait & Brenner, 1979) which indicate that epidermal LC actually represent a transient mobile pool of cells. It has been suggested that epidermal LC are replenished by indeterminate cells from the dermis (Rowden *et al.*, 1979).

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LC can be identified ultrastructurally by the presence of unique, cytoplasmic, Birbeck granules (Birbeck *et al.*, 1961) or by light microscopy using a variety of histochemical techniques (Berman & France, 1979). In addition, LC bear surface receptors for FcIgG, C3 (Stingl *et al.*, 1977) and express HLA-DR antigens (Klareskog *et al.*, 1977) in common with other macrophage populations and B lymphocytes. The recent demonstration that epidermal LC also express human thymocyte antigen (HTA-1) defined by monoclonal antibodies NA1/34 (McMichael *et al.*, 1979) and OKT6 (Fithian *et al.*, 1981) has greatly facilitated the identification of these cells *in situ*.

Increased numbers of epidermal LC may be seen in certain skin diseases (Lisi, 1973; Rowden *et al.*, 1981). Recent immunohistological studies have shown that LC may be altered in acute graft versus host disease (GvHD) following allogeneic bone marrow transplantation (manuscript in preparation). As the palms and soles are usually early sites of involvement in acute GvHD (Saurat, 1981), it is possible that this distribution may reflect regional variations in the immunobiology of normal skin.

This study was carried out to evaluate the normal distribution of cutaneous LC in various anatomical sites using monoclonal antibody NA1/34 and the standard ATPase histochemical reaction and to compare LC numbers obtained with each technique. Any regional variation in LC would clearly be important in interpreting samples of diseased skin taken from different parts of the body and may give further insight into the role of LC in various skin disorders.

Materials and methods

MATERIALS

Tissue samples

Sixty-three elliptical skin samples were obtained from 15 subjects (6 male and 9 female) with a variety of nondermatological disorders whose ages ranged from 48–87 years (Table 1). Postmortem samples were obtained from cases 1–13 and were collected within 36–72 hours of death. Fresh tissue was taken from incisional sites from patients 14 and 15 undergoing surgery. Cases 2–9 with diagnoses of malignant disease had received combination chemotherapy as well as irradiation (DXT) to the chest (cases 3–9) and cranium (case 2) for periods extending up to 2–69 months before death. Case 1 received symptomatic treatment only. Case 2 gave a history of drug allergy. Cases 10–15 had no evidence of malignant disease nor any history of drug allergy. Anatomical sites selected for biopsy were from hair-bearing scalp (12), chest (7), abdomen (12), forearm (3), dorsum of the hand (3), palm (12), dorsum of the foot (2) and sole (12).

Antisera

Monoclonal antibody NA1/34 to HTA-1 antigen was kindly supplied by Professor A. J. McMichael, Oxford. In addition to its reactivity on human cortical thymocytes, monoclonal antibody NA1/34 reacts with normal epidermal LC as well as with the abnormal cells in histiocytosis X (Thomas *et al.*, 1982). Similar reactivity patterns shown by Orthomune monoclonal antibody OKT6 suggest that both reagents react with the HTA-1 antigen through different epitopes on the same molecule which is also wholly (or partially) carried by mature LC (Thomas *et al.*, 1982). For immuno-enzyme analysis of tissue sections, monoclonal antibody, NA1/34 ascitic fluid or supernatant preparations were used at dilution 1 : 500 and 1 : 10 respectively and labelled

Table 1. Clinical data of subjects providing normal skin samples.

*Case**	*Age* (years)	*Sex*	*Diagnosis*	*Site of irradiation before sampling* (months)	*Allergic history*
1	72	Male	Lung carcinoma	—	—
2	68	Male	Lung carcinoma	C.N.S. (35)	Penicillin
3	73	Female	Lung carcinoma	Mediastinum (2)	—
4	70	Female	Lung carcinoma	Mediastinum (11)	—
5	67	Female	Breast carcinoma	Right breast (18)	—
6	62	Female	Breast carcinoma	Left breast (6)	—
7	56	Female	Breast carcinoma	Right breast (50)	—
8	54	Female	Breast carcinoma	Left breast (69)	—
9	64	Female	Breast carcinoma	Left breast (12)	—
10	75	Female	Cardiac failure	—	—
11	74	Male	Cardiac failure	—	—
12	74	Male	Chronic bronchitis	—	—
13	87	Male	Waterhouse–Friedrichson syndrome	—	—
14	65	Male	Inguinal herniorrophy	—	—
15	48	Female	Cholecystectomy	—	—

*Cases 2–9 also received varying schedules of combination chemotherapy.

with alkaline phosphatase-conjugated rabbit anti-mouse IgG antibody (Sigma; Batch 8805) at a dilution of 1 : 20.

METHODS

Tissue preparation

Skin samples (2 × 1 cm) were collected into phosphate-buffered saline (PBS), pH 7.0, and within 2 h were divided for (a) routine histology (Haematoxylin and Eosin) using modified methacarn fixation (inhibisol 30%, methanol 60%, acetic acid 10% by vol.) and paraffin wax embedding, and (b) snap freezing for vertical sections.

Preparation of frozen tissues

Vertical skin samples (1.0 × 0.5 cm) were mounted onto cork, coated with OCT (Miles Lab. Inc.) and snap frozen in isopentane pre-cooled in liquid nitrogen. These were stored in liquid nitrogen or at − 70° C until use.

Immunoalkaline phosphatase reaction

Cryostat sections (5 μm thick) were air-dried onto clean glass slides for 12 h at 22° C prior to fixation in cold (4° C) chloroform : acetone (50 : 50 vv) for 5 min. After immediate rinsing in PBS, pH 7.0 (10 min, 22° C), the sections were incubated in a damp chamber with monoclonal antibody NA1/34 (60 min, 22° C), rinsed in PBS and further incubated with alkaline phosphatase-conjugated rabbit anti-mouse IgG (60 min, 22° C). Following a final rise in PBS, the sections were incubated for 45 min to reveal the enzyme using a medium containing Naphthol AS BI phosphate, Fast Red and 1 mM levamisole to block endogenous alkaline phosphatase (Ponder & Wilkinson, 1981). The sections were briefly counterstained in fresh Haemalum (5 min)

and lithium carbonate (30 s), mounted in glycerine jelly and examined with a Leitz Laborlux 12 microscope.

ATPase histochemical reaction
Fresh 5 and 10 μm cryostat sections were fixed in cold (4° C) 0.2 M cacodylate-buffered formalin for 60 min (Robins & Brandon, 1981). Sections were immediately rinsed twice in distilled water for 5 min at 22° C and processed for the ATPase reaction using the modified method of Wachstein & Meisel as described by Lojda *et al.* (1979). Briefly, the sections were immersed in 50 ml freshly filtered incubating medium at 37° C for 60 min, washed twice in distilled water, and developed in 1% ammonium polysulphide (BDH Ltd; 10% w/v) for 3–5 min at 22° C. The incubation medium consisted of 20 mg ATP (Sigma) dissolved in equal volumes (20 ml) of distilled water and Tris–maleate buffer, pH 7.2, with 5 ml 0.1 M magnesium sulphate. Immediately prior to use, 2 ml 2% lead nitrate was added slowly (in drops) with continuous stirring. The clear solution was made up to 50 ml total volume with distilled water before filtration. After a final rinse in distilled water, the sections were mounted in glycerine jelly and examined by light microscopy.

Cell counts
Only well-orientated skin samples were evaluated. Epidermal cells (LC) positive for NA1/34 and ATPase with clearly identifiable cell bodies and dendritic processes were counted and expressed as LC counts per unit length and per unit area of epidermis.

Dimensions of the epidermis of each sample were obtained by accurately tracing the outline of the epidermal surface on to plain paper at magnification × 190 using a tracing device attached to the microscope. This device allows simultaneous visualization of both the histological section and the paper. Total length and area measurements were made by retracing these outlines on a Reichert-Jung MOP Videoplan Image Analyzer which had been calibrated for the appropriate magnification scale. The computer system was programmed to calculate the geometric parameters required for each sample automatically.

For LC counts per unit length, the total LC number was divided by the epidermal length in mm. For counts per unit area, the outline of the interfollicular epidermis was traced and the LC number divided by the epidermal area in mm^2. To enumerate LC in hair follicles, tracings were made of the outer root sheath and the area was calculated as described above.

Several important problems were encountered with the unit area measurements. Considerable variations in cell counts occurred due to variation in epidermal thickness without alteration in the LC numbers. The extremes of average epidermal thickness ranged from 15 μm in one relatively atrophic chest skin sample to 100 μm in a sample from the palm. Cell numbers per mm^2 were always considerably greater than per mm, as the average epidermal thickness was always less than 1 mm. In this study, the unit area measurements enabled us to make rough comparisons between LC in the epidermis and the hair follicles. It was not possible to achieve good orientation of the hair follicles as these were invariably cut in different planes with considerable differences in size of the outer root sheath per unit length. LC counts per mm in hair follicles were, therefore, considered inappropriate.

Statistical analysis
In view of the marked variation in LC counts from subject to subject, the data was transformed logarithmically. The ratio of the values at different sites (rather than their differences) were considered, and the ratio was summarized by the geometric rather than the arithmetic mean. The statistical significance of results was calculated by the paired *t*-test on the transformed data. Owing to the problems of calculating LC per unit area, statistical analysis was confined to LC counts obtained per unit length.

Results

All skin samples were histologically normal.

HTA-1⁺ LC in normal skin

Large HTA-1$^+$ LC could be clearly identified by the distinctive red alkaline phosphatase reaction product in 62 out of 63 skin samples and the staining intensity was equal on all positive cells. In contrast, no HTA-1$^+$ activity was observed on epidermal keratinocytes, melanocytes or scanty lymphocytic populations in the dermis.

The highly dendritic LC were found mainly (but not exclusively) in the suprabasal and middle layers of the epidermis. Sparse perivascular accumulations were also present in the superficial dermis in occasional samples from hair-bearing skin (Fig. 1) but not in those from the palm or sole. The morphology was generally similar in all sites although the cell processes were often more poorly developed in the palm and sole (Fig. 2). In addition, a striking feature of hair-bearing skin was the presence of conspicuous numbers of LC within the outer root sheath of hair follicles (see below).

ATPase$^+$ LC in normal skin

The ATPase reaction was optimally demonstrated on 10 μm sections (Fig. 3) and showed epidermal LC populations identical to those identified by monoclonal antibody NA1/34. This was established by superimposing alkaline phosphatase immunocytochemistry (red) on sections previously stained for ATPase (brown). All ATPase$^+$ epidermal and scanty ATPase$^+$ dermal LC expressed HTA-1 antigen. However, ATPase$^+$, HTA-1$^-$ dendritic cells were identified in the superficial dermis of most skin samples. Other dermal structures with intense ATPase activity included capillary endothelium (Fig. 3) and sebaceous glands but the lymphocyte populations were ATPase

Epidermal LC counts in different skin sites

The results are summarized in Table 2 and Fig. 5. LC counts in different anatomical sites showed marked variation between individual cases. Despite these variations, consistently high numbers were obtained in skin samples from the chest (mean 16.2 per mm), scalp (mean 11.5 per mm) and abdomen (mean 9.9 per mm), whereas low numbers were always enumerated in the palm (mean 2.3 per mm) and sole (mean 2.5 per mm) samples. There were no statistical differences between the mean values obtained for the chest, scalp and abdomen ($P > 0.10$) or for the mean values from the palm and sole ($P > 0.5$). However, when the combined values for palm and sole were compared with those obtained for chest, scalp and abdomen, a highly significant difference emerged ($P < 0.001$). On average, the hair-bearing skin samples contained about six times as many LC per mm as those from non-hair-bearing sites (Fig. 5).

A separate comparison was made between the combined values for palm and sole, and the combined values of the anatomically close dorsum of hand and dorsum of foot. Again, a significant difference was observed ($P < 0.05$; Table 2).

A similar comparison was made between cases with and without malignant diseases

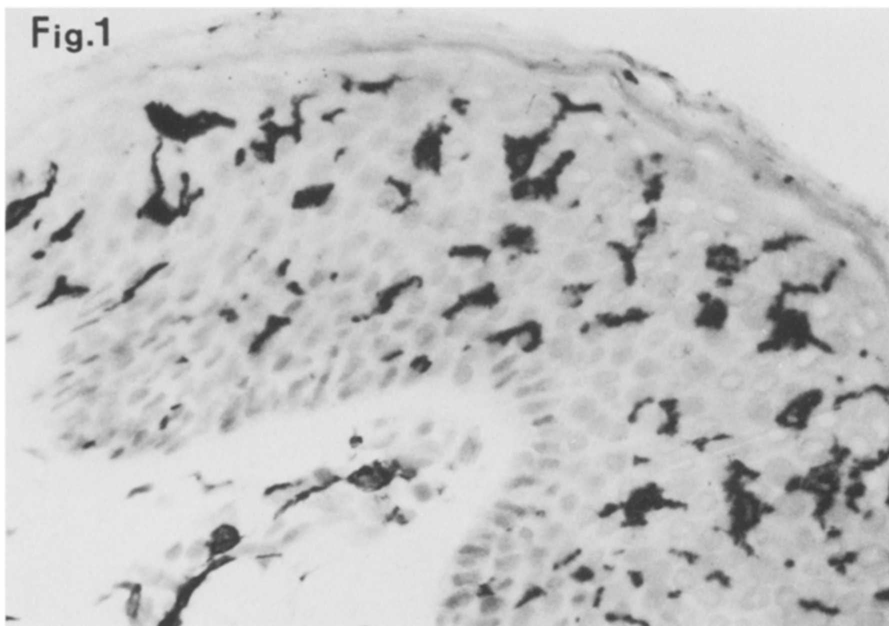
Fig.1

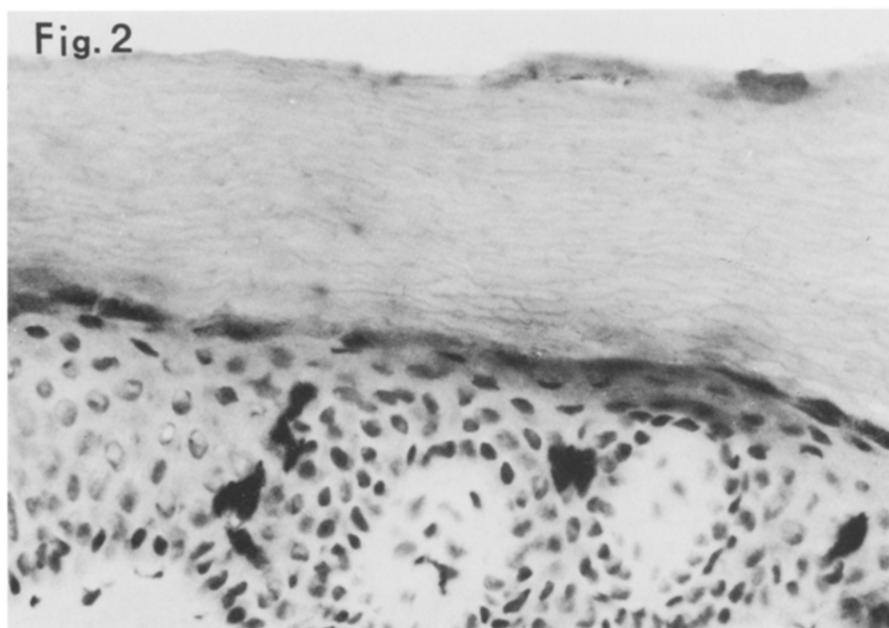
Fig. 2

Table 2. Range and mean values of LC numbers in normal human skin at different anatomical sites.

Site	*Number of samples* (cases)	*LC per unit length* (mm) (mean)	*LC per unit area* (mm^2) (mean)
Palm	12	0.4–7.0	5.4–58.5
	(1–9, 11–13)	(2.3)	(25.9)
Sole	12	0–8.9	0–113.2
	(1–11, 13)	(2.5)	(37.7)
Chest	7	0.4–35.1	9.8–1281.8
	(1, 2, 4, 6–9)	(16.2)	(593.8)
Abdomen	12	2.9–30.3	104.3–566.6
	(1, 2, 4, 6–12, 14, 15)	(9.9)	(254.2)
Scalp	12	1.7–23.9	50–888.23
	(1–4, 6–13)	(11.5)	(305.2)
Forearm + dorsum of hand	6	0.54–10.9	11–306.6
	(6–8, 10, 12, 13)	(6.3)	(164.8)
Dorsum of foot	2	4.3–4.6	66.6–125.8
	(10, 13)	(4.4)	(96.2)

Statistical analyses (paired t-test)
Palm *versus* sole: $t = 0.7$; $P > 0.5$
Chest *versus* abdomen *versus* scalp: $t = 1.2$; $P > 0.1$
Palm/sole *versus* forearm + dorsum of hand/dorsum of foot: $t = 2.81$; $P < 0.05$
Cancer cases (2–9) *versus* non-cancer cases (10–15): $t = 1.26$; $P > 0.1$

in order to determine any bias due to the effects of radiotherapy and/or chemotherapy. No significant difference in mean LC values was observed between the two groups of subjects (Table 2).

LC counts in hair follicles

LC were confined to the outer root sheath (Fig. 4), and became less numerous in the deeper portions. The LC density in the superficial portions of the follicles was not significantly different from that in the epidermis (Table 3). An impression of higher LC

Fig. 1. Cryostat section (5 μm) of normal human skin from the anterior abdominal wall stained indirectly with monoclonal antibody (NA1/34) to HTA-1 antigen and rabbit anti-mouse IgG alkaline phosphatase conjugate. Numerous highly dendritic HTA-1$^+$ LC are present throughout the epidermis. Scanty perivascular HTA-1$^+$ LC are also demonstrated in the superficial dermis. × 315.

Fig. 2. Cryostat section (5 μm) of normal human skin from the palm stained indirectly with monoclonal antibody (NA1/34) to HTA-1 antigen and rabbit anti-mouse IgG alkaline phosphatase conjugate. Low numbers of HTA-1$^+$ LC with short dendritic processes are exclusively situated in the epidermis. × 315.

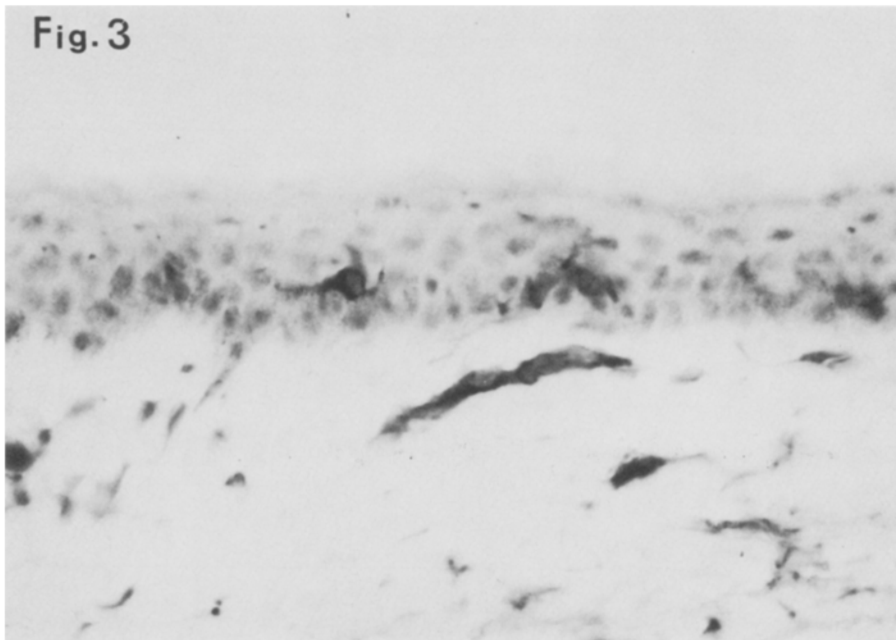
Fig. 3

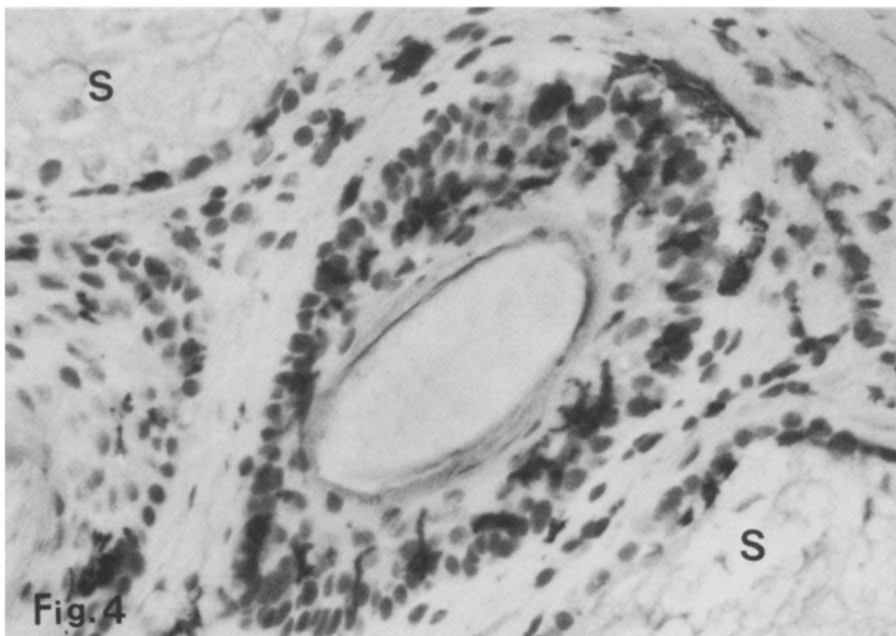
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Fig. 4

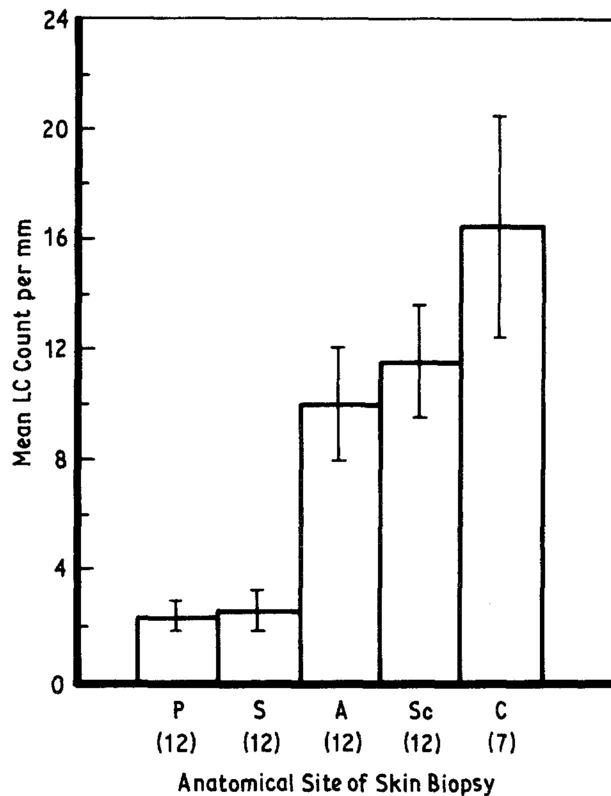

Fig. 5. Distribution of (mean) epidermal LC counts per mm in normal human palm (P), sole (S), anterior abdominal (A), scalp (Sc) and anterior chest (C) skin. The difference between LC densities in hairless (P + S) and hair-bearing (A + Sc + C) skin is statistically significant ($t = 7.76$; $P < 0.001$: paired t-test). The number of samples is given in parentheses.

numbers in the follicles was produced by a tangential cut through the outer root sheath. This gave a view of the follicle similar to that obtained with a horizontal cut through the epidermis.

A small number of LC was also identified in the peripheral portions of sebaceous glands (Fig. 4) but were not demonstrated in sweat glands or any other adnexal structure.

Fig. 3. Cryostat section (10 μm) of normal human skin from the anterior chest wall stained with ATPase. ATPase$^+$ LC with long processes are demonstrated in the supra-basal region of the epidermis. Blood vessel endothelial cells in the superficial dermis also show strong ATPase activity. $\times$ 315.

Fig. 4. Cryostat section of normal human skin from the scalp stained indirectly with monoclonal antibody NA1/34 to HTA-1 antigen and rabbit anti-mouse IgG alkaline phosphatase conjugate. Sebaceous glands (S) define the middle portion of a hair follicle which contains numerous LC in the outer sheath. $\times$ 315.

Table 3. LC count per mm^2 in superficial, middle and deep portions of hair follicles from 12 scalp samples.

Portion of hair follicle	*Total number of hair follicles*	*LC count per* (mm^2) (mean)
Superficial	37	0–1600 (545)
Middle	92	35–454 (135)
Deep	37	0–38.8 (4)

Discussion

In this study, LC populations in histologically normal skin from different anatomical sites were identified by the immuno-alkaline phosphatase technique using monoclonal antibody to HTA-1 antigen and by histochemical labelling with ATPase. The morphology and location of LC were well demonstrated by both markers. ATPase has been traditionally the most convenient means of identifying LC (Mackenzie & Squier, 1975; Juhlin & Shelley, 1977; Robins & Brandon, 1981), but more recently, the specific expression of HTA-1 antigen on LC has been visualized by immunohistological techniques at the light and electron microscope levels (Murphy *et al.*, 1981; Chu *et al.*, 1982). By using combined immunological and histochemical labelling techniques in this investigation, both ATPase and monoclonal antibody to HTA-1 antigen were shown to react with identical populations of LC. The uniform expression of HTA-1 antigen and ATPase throughout each cell contrasts with α-D-mannosidase activity (Elleder *et al.*, 1977) which selectively reveals the LC body and excludes the dendritic processes (B. Lake, personal communication).

Previous studies have used a variety of immunological and histochemical techniques to enumerate LC in skin. Many of these have employed epidermal sheet preparations in which higher LC counts per unit area are obtained as the cells are visualized horizontally in the full thickness of the epidermis rather than in the vertical plane. The LC values calculated in the present study, therefore, cannot be compared to the results of investigations using epidermal sheets. Epidermal sheet preparations are probably the most accurate way to enumerate LC but they do not allow simultaneous assessment of histopathological abnormalities in the samples. This is an important point as the present study is intended to form a baseline for future investigations of various skin abnormalities.

Variation in LC populations in different anatomical sites have been observed (but not enumerated) in normal rodent and human skin using immunofluorescence with heterologous antibody to HLA-DR antigens (Nordlund & Ackles, 1981) and ATPase (Juhlin & Shelley, 1977). Quantitative studies on epidermal sheets of normal rodent skin showed low numbers of ATPase$^+$ LC in parakeratotic regions of mouse tail

compared to orthokeratotic skin in other sites (Schweizer & Marks, 1977; Bergstresser *et al.*, 1980). By contrast, other studies have shown virtually no differences in LC populations in rodent (Wolff & Winkelmann, 1967) or normal human skin (Brown *et al.*, 1967; Riley, 1967; Lisi, 1973). The presence of conspicuous numbers of LC in the outer root sheath of hair follicles and variable numbers associated with sebaceous glands has been previously shown (Jimbow *et al.*, 1969) but variation in LC densities at different levels of the dermis was not noted.

Depletion of LC can be produced by ultraviolet light (Toews *et al.*, 1980), X-ray irradiation (Fan *et al.*, 1959) and sunlight (Zelickson & Mottaz, 1968). Although there is a temporary state of unresponsiveness to contact allergens due to LC depletion, these numerically and functionally return to normal following withdrawal of radiation exposure. This further suggests that LC belong to a mobile population and supports our present finding of normal LC numbers in previously X-ray irradiated patients. Similarly, the LC within the pilo-sebaceous units were either unaffected by the transient alopecia produced in the patients' cytotoxic chemotherapy, or were renewed along with hair regrowth. Exposure to sunlight is clearly not the explanation for the lower LC values in hairless skin. In the subjects studied, the sole was not a sun-exposed site and considerably greater numbers of LC were found in skin from the dorsum of the hand and foot.

The problems of quantifying LC have been discussed above. Moreover, LC counts could vary from one laboratory to another even if similar techniques are used, due to problems of observer variation, section thickness and staining intensity. Clearly, careful morphometry and statistical analysis are necessary as well as identification of the anatomical sites of the samples under study. The results show a substantial difference in LC numbers between hair-bearing and hairless skin but no statistical difference between the hair-bearing sites. There was, however, a marked variation in the results between the individual subjects. Our subsequent studies have shown relatively higher and more constant LC values in surgical biopsies removed from the same site in different cases suggesting that postmortem changes may contribute to the variation in hair-bearing sites. A postmortem study, however, has the advantage of allowing samples to be taken from multiple sites in the same subject. Demonstration of significant differences in LC at different anatomical sites are clearly important for establishing normal baseline values in any quantitative study of LC in pathological conditions.

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