# HUMAN EPIDERMAL T CELLS PREDOMINATELY BELONG TO THE LINEAGE EXPRESSING $\alpha/\beta$ T CELL RECEPTOR

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Normal human epidermis, long since dismissed as merely a protective covering for the body, is now recognized as a complex immunological unit. This concept is based on the demonstration that Langerhans cells (LCs)[1] are potent APCs for a variety of T cell (TC) responses and that keratinocytes (KC), at least upon stimulation, can secrete a plethora of immunomodulating cytokines (reviewed in reference 1). Finally, it has been known for almost 40 years that the normal human epidermis harbors lymphocytic-appearing cells (2), yet surprisingly this cell population has received little attention since then. The relatively recent discovery of a TCR-$\gamma/\delta$-bearing lymphocyte population (DETC, dendritic epidermal T cell) in the mouse epidermis (reviewed in reference 1) has rekindled interest in the possibility not only that the epidermis may function as an extrathymic microenvironment for the differentiation and/or "education" of TCs but also that a resident TC population may exist in the human epidermis, particularly an equivalent to the murine DETC. While some investigators never, or only rarely, observed epidermal TCs in normal skin (3, 4), we and others have found them to be routinely detectable in nonlesional, clinically normal skin (5–9). In this study we have sought to (*a*) more fully describe the heterogeneous phenotype of epidermal TCs, particularly with regard to TCR-$\alpha/\beta$- and -$\gamma/\delta$-bearing subpopulations, (*b*) examine ultrastructural features of immunolabeled epidermal TCs, (*c*) quantitate this population in various body regions, (*d*) examine their in

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[1] *Abbreviations used in this paper:* ABC, streptavidin-biotin-complex; CD, cluster of differentiation; DAB, 3',3'-diaminobenzidine; DETC, dendritic epidermal T cell; EC, epidermal cell(s); GAM, goat anti-mouse; IIF, indirect immunofluorescence; IIP, indirect immunoperoxidase; KC, keratinocyte(s); LC(s), Langerhans cell(s); NGS, normal goat serum; RT, room temperature; SALT, skin-associated lymphoid tissues; TC(s), T cell/lymphocyte(s); WM, washing medium.

situ relationship to LCs (*e*) investigate the growth potential of epidermal TCs in vitro, and (*f*) characterize the phenotype and CD3-associated TCR complex of cultured epidermal TCs by FACS analysis and immunoprecipitation.

## Materials and Methods

### *Specimen Collection*

Full- or split-thickness skin biopsies from clinically normal-appearing areas were obtained during elective surgery under local anesthesia and from autopsy specimens using a dermatome (Davol/Simon, Cranston, RI). Skin from 50 individuals was examined, and for some aspects of this study, represented six different body regions (i.e., ventral thigh and upper arm, chest, buttock, back, sole [posterior to "ball of foot"]).

### *Preparation of Tissue*

*Frozen Sections.* Using standard techniques, full-thickness skin biopsies were snap frozen in a liquid nitrogen bath and embedded in OCT compound (Miles Laboratories, Naperville, IL). 5-$\mu$m-thick sections were acetone-fixed for 10 min at 4°C.

*Epidermal Sheets.* Keratomed, split-thickness skin (sole or thigh) was rinsed in washing medium (WM: = RPMI 1640 [Gibco Ltd., Paisley, Scotland] supplemented with 10% FCS (Flow Laboratories, Ltd., Ayrshire, Scotland], 2 mM glutamine [Flow], 25 mM Hepes buffer [Flow], 50 $\mu$g/ml gentamicin [Flow], and an antibiotic-antimycotic solution [Gibco] consisting of 100 $\mu$g/ml penicillin, 100 $\mu$g/ml streptomycine, 0.25 $\mu$g/ml Fungizone), then cut into 3–4-$cm^2$ pieces. Epidermal sheets were prepared by using either the ammonium thiocyanate separation technique (10) or 3 U/ml dispase (Boehringer-Mannheim, Mannheim, FRG), as previously reported (11).

*Epidermal Cell Suspensions.* Under sterile conditions, keratomed skin was incubated for 1 h at room temperature (RT) in WM additionally supplemented with a 10× concentration of the antibiotic/antimycotic solution, followed by a wash in PBS without calcium and magnesium (Dulbecco's PBS), pH 7.6. Epidermal sheets were prepared using either the dispase method (11) or PBS with 0.25% trypsin (Gibco Ltd.) for 1 h at 37°C, then these were placed in WM containing 0.025% deoxyribonuclease I (Sigma Chemical Co., St. Louis, MO). After 20–30 min at RT the sheets were vigorously stirred, and the resulting cell suspension was poured through a sterile nylon gauze sieve to remove large clumps of cells. Viability was routinely 70–90%, as determined by trypan blue exclusion.

*Enrichment of Epidermal T Cells.* Preliminary observations showed that unfractionated epidermal cell (EC) suspensions contain only minute numbers of TCs. Therefore, to increase the percentage of TCs within the EC suspension, and thus improve the chances of stimulating their proliferation in vitro, we used a technique that results in the elimination of most KC and also leads to a substantial enrichment of LCs (Yokozeki et al., manuscript in preparation). In brief, EC suspensions were incubated for 1 h at 37°C in tissue culture flasks that had been coated with Vitrogen 100 (Collagen Corp., Palo Alto, CA). Nonadherent cells were resuspended in ice-cold PBS with simultaneous vortexing. This treatment selectively killed the majority of KC, which were subsequently removed by density gradient centrifugation. The interface cells (enriched for TCs) were collected and prepared for the appropriate experiments, as described below.

### *Immunolabeling Procedures*

Staining was performed by using either indirect immunofluorescence (IIF), indirect immunoperoxidase (IIP), or a peroxidase-conjugated ABC (streptavidin-biotin-complex) technique. In each method, irrelevant mAbs of the appropriate Ig isotype were used as negative controls. To assay for endogenous peroxidase activity, both primary and secondary antibodies were omitted from the procedure.

*Frozen Sections.* Regardless of the staining method (IIF, IIP, or ABC), sections were preblocked in 10% normal goat serum (NGS) for 30 min at RT then incubated in the primary mAb (Table I) overnight at 4°C or 6 h RT, followed by a PBS wash and another 30 min incubation in 10% NGS. For IIF, the sections then were incubated in a 1:30 dilution of either

TABLE I
*mAbs for Immunolabeling Epidermal Sheets, Cell Suspensions and Frozen Sections*

| Cluster of differentiation | mAb used* | Source‡ | IgG subclass | Antigen specificity/cellular expression |
|---|---|---|---|---|
| CD1a | OKT6 | O | IgG1 | T6/cortical thymocytes; LCs |
| CD2 | Leu 5b | BD | IgG2a | T11/sheep erythrocyte rosette receptor; pan TC |
| CD3 | Leu 4 | BD | IgG1 | T3 complex antigen |
| CD4 | Leu 3a | BD | IgG1 | T4/helper-inducer TC |
| CD5 | Leu 1 | BD | IgG2a | pan TC |
| CD7 | Leu 9 | BD | IgG2a | pan TC |
| CD8 | Dako-T8 | D | IgG1 | T8/suppressor-cytotoxic |
| | Leu 2a | BD | IgG1 | TC |
| CD14 | Leu M3 | BD | IgG2b | Monocytes/macrophages |
| CD16 | Leu 11a | BD | IgG1 | IgG Fc receptor on NK cells and neutrophils |
| CD19 | Leu 12 | BD | IgG1 | B cells (B4) |
| CD20 | Leu 16 | BD | IgG1 | B cells (B1) |
| CD25 | Dako IL2R | D | IgG1 | IL-2 receptor (Tac antigen) |
| | α-IL2R | BD | IgG1 | on activated TCs |
| CD29 | 4B4 | C | IgG1 | Subset of B and TCs |
| CD45 | HLe-1 | BD | IgG1 | Pan leukocyte |
| CD45 | Dako-LC | D | IgG1 | Pan-leukocyte, except for LCs |
| CD45RA | 2H4 | C | IgG1 | Unsensitized TCs |
| CD45RO | UCHL1 | D | IgG2a | Memory TCs |
| – | VLA-1 | TS | IgG1 | TC late activation antigen |
| – | HLA-DR | BD | IgG2a | Class II MHC antigen |
| – | WT31 | BD | IgG1 | TCR-α/β |
| | BMA031 | B | IgG2b | TCR-α/β |
| | βF1 | TS | IgG1 | TCR β chain, framework |
| – | δTCS1 | TS | IgG1 | TCR Vδ1 |
| – | TCRδ1 | TS/MB | IgG1 | TCR δ chain, framework |

* Mouse anti-human mAb, unless designated otherwise.
‡ BD, Becton Dickinson; B, Behring; C, Coulter; D, Dako; MB, gift from M. Brenner (Boston, MA); O, Ortho Diagnostics; TS, T Cell Sciences.

rhodamine-conjugated goat anti-mouse (GAM) IgG (Atlantic Antibodies, Scarborough, ME) or FITC-conjugated $F(ab')_2$ GAM IgG (Tago Inc., Burlingame, CA) for 1-2 h at RT, washed in PBS, and coverslipped in mounting fluid (Difco Laboratories, Detroit, MI).

In the ABC method, sections were incubated in a 1:200 dilution of biotinylated sheep anti-mouse IgG (Amersham International, Amersham, U. K.) for 1-2 h at RT, washed in PBS, then treated with the peroxidase-ABC solution (Dakopatts a/s, Glostrup, Denmark) for 1 h at RT, according to manufacturer's instructions. Finally, sections were incubated in 3-amino-9-ethylcarbazole (Sigma) for 5-10 min, then washed, counterstained with hematoxylin, and coverslipped in Aquamount (BDH Ltd., Poole, UK).

For IIP staining, sections were incubated in a 1:100 dilution of peroxidase-conjugated sheep anti-mouse IgG (Amersham) for 1-2 h at RT, washed in PBS, and subjected to the 3',3'-diaminobenzidine (DAB) reaction.

*Epidermal Sheets.* Sheets were fixed in either acetone for 3 min at RT or in Nakane's fixative (12) for 1 h at RT. The sheets were stained by either the IIF or IIP technique (as described above for frozen sections), then coverslipped (basal surface facing up) in mounting fluid (Difco).

*Double Labeling of Frozen Sections and Epidermal Sheets.* Double labeling of either sections or epidermal sheets was performed using an IIF technique (as described above, with the rhodamine conjugate) for visualization of the first mAB, followed by a 1 h incubation at RT in 10% normal mouse serum and 10% NGS. Specimens then were stained with a FITC-conjugated mAb, usually at high concentrations (1:10), for a 2–4 h at RT, then washed.

*Cell Suspensions.* Cultured TCs, and EC suspensions enriched for TCs, were immunolabeled with a battery of mAbs (Table I) by a standard IIF technique using FITC-conjugated $F(ab')_2$ GAM IgG (Tago) as the second antibody for 15 min at 4°C. For negative controls, the primary mAb was substituted by either PBS or irrelevant antibodies of the appropriate subclass. Cells were either examined with a fluorescence microscope or analyzed on a FACS Analyzer (BD, Sunnyvale, CA), using 10,000 cells/sample. Nonviable cells were gated out by propidium iodide staining.

*Transmission Electron Microscopy.* Epidermal sheets or thin slices of skin (80–100 μm thick) were immersed in Nakane's fixative (12) for 1 h at RT, washed in PBS/7.5% sucrose, then preblocked in 10% normal horse serum for 1 h at RT. The specimens were incubated in the primary mAb (diluted in PBS/sucrose) for 6–12 h at 4°C. After washing in PBS/sucrose, the tissue was incubated in a 1:50 dilution of biotinylated horse anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA) for 4–6 h at 4°C, rinsed, and incubated in a 1:100 dilution of a peroxidase-conjugated ABC solution (Vector) for 4–6 h at 4°C. The specimens were immersed in half-strength Karnovsky's fixative (13) for 30 min at 4°C, washed in 0.2 M cacodylate buffer, then incubated in 0.02% DAB solution for 30 min at RT. The tissue was post-fixed in 2% $OsO_4$-potassium ferrocyanide for 1 h at 4°C and processed for Epon-embedment. Thin sections were lightly stained, as previously described (14), and examined with a JEOL 1200EX electron microscope.

### *Quantitation Method*

To use a large panel of mAbs on each individual skin specimen and to evaluate staining patterns in both the epidermis and dermis, all of the quantitative data presented in this study were derived from cryostat sections and compared in five body regions (Table II), i.e., chest [5]; buttock [5]; sole [8]; and limbs, e. g., thigh [2] + upper arm [3]. The mean number of peroxidase-labeled cells ± SD was calculated in three to six sections per specimen, using a 40× objective, and normalized for the number of cells per linear centimeter of epidermis. Only clearly stained cell bodies were counted.

### *Statistical Analysis*

The mean number of TCs/linear cm of epidermis does not necessarily conform to the definition of a normally-distributed population. Therefore a non-parametric analysis of variance, using the Kruskal-Wallis test (15), was employed to determine whether there were differences between the number of epidermal $CD2^+/CD3^+$ cells within the four treatment groups (thorax, limbs, buttock, sole). Observed differences were analyzed using the Mann-Whitney U-test (16) and were considered significant if $p \leq 0.05$.

### *Culture of Epidermal T Cells*

EC suspensions enriched for TCs (as well as LCs) were incubated in complete culture media (WM with 1% nonessential amino acids [Flow] and 100 mM sodium pyruvate [Flow]) supplemented with 5 μg/ml Con A (Pharmacia Fine Chemicals, Uppsala, Sweden), 40 U/ml rIL-1 (Genzyme Corp., Boston, MA), 100 U/ml rIL-2 (Amersham), and 10% A431 cell culture supernatant (concentrated 10× from a 3-d subconfluent population, using an Amicon filter [Amicon Ltd., Stonehouse, UK]). The cell suspensions were seeded at a high density in appropriate tissue culture plates/flasks and incubated at 37°C in 5% $CO_2$.

### *Immunoprecipitation and SDS-PAGE Analysis*

Cells cultured from TC-enriched EC suspensions were investigated for their TCR configuration using an immunoprecipitation technique with mouse anti-human CD3δ and rabbit anti-human TCR-α, anti-human TCR-β, anti-human TCR-γ antibodies, as described by Koning and colleagues (17, 18).

TABLE II

*Regional Density of Epidermal TC*

| Specimen number (region) | Panel of mAbs | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CD2 | CD3 | CD5 | CD7 | CD4 | CD8 | TCR-α/β | TCR-γ/δ | CD25 | VLA-1 | 2H4 | UCHL1 |
| 01 (l) | 2.0 | 5.8 | 7.1 | 0 | 2.5 | 3.5 | 0.7 | 0.3 | 0 | 0 | 0 | 1.4 |
| 02 (l) | 3.6 | 2.4 | 2.7 | 0.9 | 0 | 2.2 | 1.3 | 0 | 0.5 | 1.3 | 2.7 | 0.7 |
| 03 (l) | 5.4 | 6.7 | 6.5 | 3.4 | 3.7 | 4.8 | 5.2 | 0 | 2.8 | 0.9 | 4.3 | 4.3 |
| 04 (l) | 3.3 | 3.3 | 0 | 3.3 | 1.6 | 0 | 0 | 0 | 1.6 | 0 | – | – |
| 05 (l) | 15.0 | 11.6 | 4.1 | 8.3 | 3.3 | 1.6 | 6.6 | 0 | 3.3 | 0 | 0.5 | 12.7 |
| 06 (t) | 5.0 | 6.8 | 4.0 | 3.2 | 0.9 | 0 | 1.1 | 0.5 | 2.5 | 0 | 0 | 5.9 |
| 07 (t) | 15.0 | 18.3 | 10.0 | 3.3 | 5.0 | 3.3 | 4.1 | 0 | 1.6 | 8.3 | 0 | 0.7 |
| 08 (t) | 22.5 | 21.6 | 21.6 | 2.5 | 8.7 | 5.8 | 20.8 | 0 | 2.5 | 0.8 | 0.8 | 19.1 |
| 09 (t) | 10.0 | 8.3 | 7.9 | 2.5 | 2.9 | 1.7 | 1.3 | 0.4 | 2.9 | 0.4 | 0.8 | – |
| 10 (t) | 11.6 | 16.6 | 8.3 | – | 0 | 1.7 | 2.2 | 0 | 3.3 | 0 | 0 | 7.2 |
| 11 (b) | 4.1 | 15.0 | 5.8 | 5.8 | 2.5 | 2.5 | 0 | 0 | 2.5 | 0 | 4.1 | 10.0 |
| 12 (b) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.5 |
| 13 (b) | 8.7 | 15.0 | 13.7 | 2.5 | 2.5 | 16.2 | 0 | 0 | 2.5 | 0 | 1.7 | 7.5 |
| 14 (b) | 1.0 | 3.1 | 3.7 | 0 | 1.3 | 0.6 | 0 | 0 | 2.6 | 0 | 2.5 | 3.3 |
| 15 (b) | 7.5 | 10.0 | 10.0 | 2.5 | 2.5 | 5.0 | 0 | 0 | 12.5 | 0 | 0 | 2.5 |
| $\bar{x}$ = | **8.3** | **9.6** | **7.0** | **2.7** | **2.5** | **3.3** | **2.9** | **0.08** | **2.7** | **0.8** | **1.2** | **6.0** |
| ± SD | **±6.5** | **±6.3** | **±5.3** | **±2.2** | **±2.1** | **±3.9** | **±5.2** | **±0.2** | **±2.8** | **±2.1** | **±1.5** | **±5.2** |
| 16 (s) | 30.0 | 30.0 | 36.1 | 3.0 | 23.0 | 15.0 | 11.5 | 0 | 0 | 4.7 | 24.0 | 10.7 |
| 17 (s) | 80.0 | 70.0 | 81.2 | 12.5 | 32.5 | 46.2 | 20.0 | 0 | 0 | 1.2 | 1.2 | 65.2 |
| 18 (s) | 41.2 | 42.5 | 45.0 | 8.7 | 18.7 | 33.7 | 11.8 | 0 | 0 | 3.7 | – | – |
| 19 (s) | 31.0 | 30.0 | 32.0 | 13.3 | 10.0 | 23.4 | 29.0 | 0 | 0 | 2.0 | 10.6 | 22.0 |
| 20 (s) | 43.4 | 47.3 | 24.7 | 10.5 | 13.8 | 33.8 | 10.5 | 1.2 | 0 | 5.0 | 4.0 | 19.0 |
| 21 (s) | 33.0 | 45.0 | 26.0 | 27.0 | 11.0 | 23.5 | 27.5 | 0 | 0 | 0 | 6.0 | 26.0 |
| 22 (s) | 33.8 | 35.0 | 24.9 | 3.9 | 19.8 | 35.3 | 34.6 | 0 | 0.8 | 2.1 | 2.5 | 37.8 |
| 23 (s) | 54.0 | 46.0 | 46.0 | – | 22.5 | 23.0 | 40.0 | 1.0 | 0 | 3.0 | 6.0 | 20.0 |
| $\bar{x}$ = | **43.3** | **43.2** | **39.5** | **11.3** | **18.9** | **29.2** | **23.1** | **0.3** | **0.1** | **2.7** | **7.8** | **28.7** |
| ± SD | **±15.8** | **±12.1** | **±17.7** | **±7.4** | **±6.9** | **±9.2** | **±10.6** | **±0.5** | **±0.3** | **±1.6** | **±7.2** | **±16.7** |

Using a panel of mAbs (see Table I), epidermal TCs were immunolabeled and counted in four body regions (b, buttock; l, upper and lower limbs; s, sole; t, thorax); skin from five to eight different individuals was examined in each of these regions, with a total of 23 specimens. Data are shown as number of cells/linear centimeter of epidermis. Where there was no difference ($p > 0.1$) in TC density between regions (i.e., limbs, thorax and buttock), the data are collectively expressed as mean ($\bar{x}$) number of cells + / − standard deviation (SD). Plantar TC numbers, significantly greater than the other regions, are shown as a separate group. −, data not available.

## Results

A heterogeneous population of hematopoietic cells ($CD45^+$) was observed in the epidermis of clinically normal-appearing skin from almost every specimen examined. Double immunolabeling with anti-CD45 and either anti-CD1a, anti-CD2, or anti-CD3 mAbs (see Table I) demonstrated two discrete subpopulations of $CD45^+$ cells (Fig. 1). The majority were $CD1a^+/HLA\text{-}DR^+$, highly dendritic, suprabasally located cells, which by definition corresponded to LCs. $CD1a^+$ cells were occasionally $UCHL1^+$, which to our knowledge is a new but not surprising observation, and frequently $CD4^+$. However, LCs did not express CD2, CD3, CD5, CD7, or CD8 antigens (we therefore can not substantiate the previously reported occurrence [19] of CD3-associated TCR moieties on $CD1a^+$ epidermal cells). Furthermore, LCs also were unreactive with the mAb Dako LC (20) (product name refers to "leukocyte common" antigen and not to Langerhans cell), which supposedly belongs to the CD45 cluster; thus, in our opinion the Dako-LC reagent should not be considered as a classical anti-CD45 mAb.

The other epidermal $CD45^+$ population (Fig. 1 *b*) consisted of $CD1a^-/CD2^+/CD3^+$/Dako $LC^+$ cells that were smaller than LCs, truncate or slightly dendritic, and located in either the basal or suprabasal layer. Neither $CD2^+/CD3^-$ nor $CD2^-/CD3^+$ cells were encountered within the epidermis. Monocytes/macrophages, natural killer cells, and B cells also were not observed within the epidermal compartment, as judged by lack of reactivity with anti-CD14, -CD16 and -CD19/20 reagents, respectively. Therefore, throughout this study we will refer to the $CD45^+/CD1a^-/CD2^+/CD3^+$ population within the epidermis as T lymphocytes, in accordance with our present state of knowledge regarding TC differentiation antigens; the mean number of TCs exhibiting this phenotype will be used as a reference point in comparing their density with that of other immunolabeled cells.

*Phenotype, Distribution, Regional Variability, and Density of Epidermal TCs.* Epidermal TCs (i.e., $CD2^+/CD3^+$) were almost exclusively $CD5^+$ (Table II). They were distinguishable from LCs not only phenotypically but also morphologically, since TCs had a more spherical nuclear profile with a higher nuclear-to-cytoplasmic ratio, lacked Birbeck granules, and exhibited a denser cytoplasm due to the abundance of free ribosomes and nonaggregated, intermediate-size filaments (Fig. 2). Also, TCs usu-

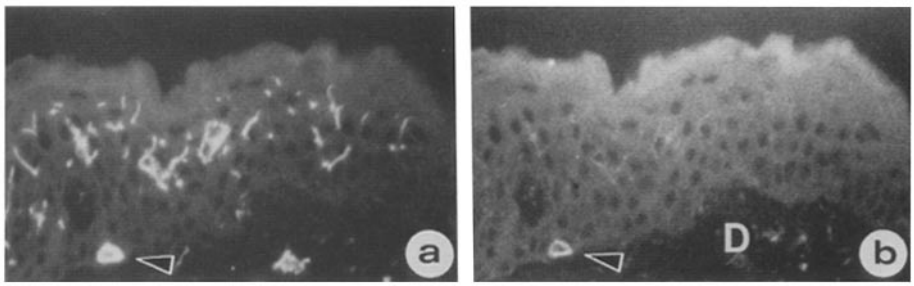


FIGURE 1. Double immunostain of rhodamine-labeled $CD45^+$ (*a*) and FITC-labeled $CD3^+$ (*b*) epidermal cells in a frozen section of back skin. A $CD45^+/CD3^+$ TC is depicted within the basal KC layer (arrowhead), while LCs represent the majority of $CD45^+$ cells. In every double-labeling experiment, $CD3^+$ TCs coexpressed CD2 antigens (photograph not included). D, dermis. ×120.

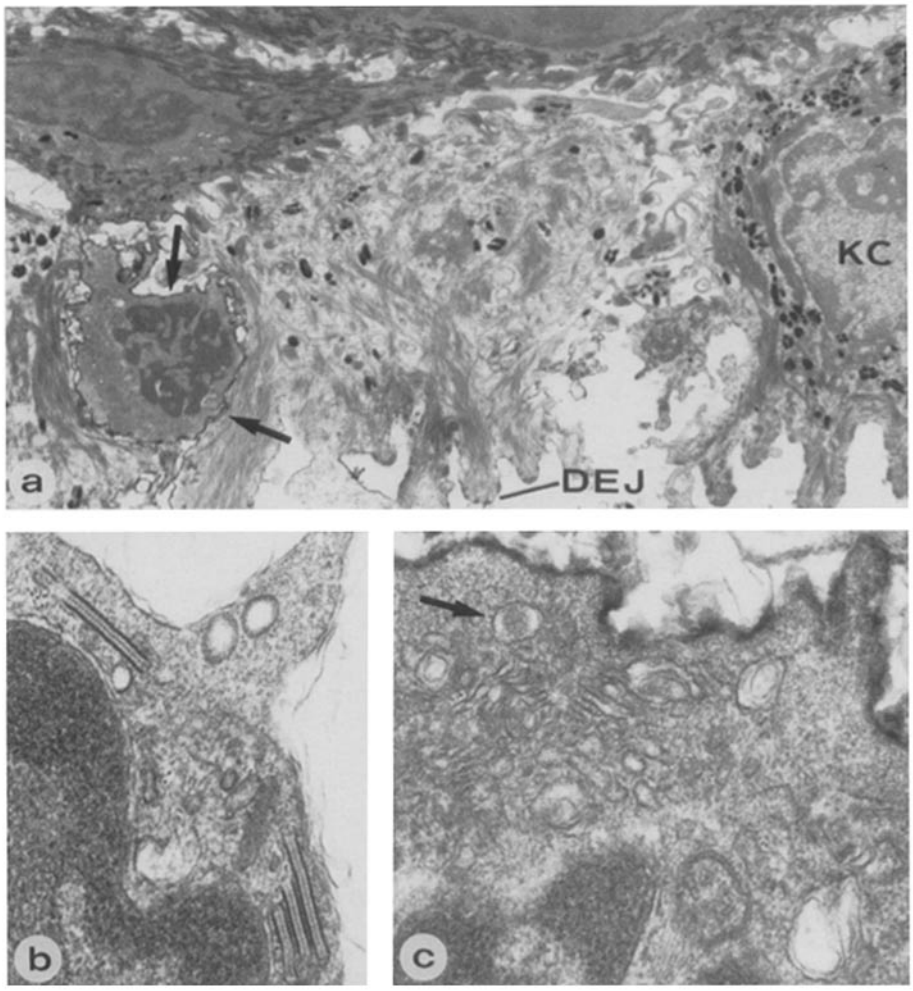


FIGURE 2. Three electron micrographs of an epidermal sheet from the thigh, immunolabeled with anti-Leu 1. (*a*) Low power view of a peroxidase-labeled CD5$^+$ TC (arrows) within the basal KC layer; DEJ, dermal-epidermal junction. ×7,000. (*b*) Portion of an adjacent LC showing Birbeck granules, and as expected, no CD5 reactivity; ×45,000. (*c*) Higher magnification of another CD5$^+$ TC-exhibiting peroxidase reaction product along the plasma membrane and a typical cytoplasmic granule (*arrow*). ×47,300.

ally contained a few small cytoplasmic granules that were round to ovoid with a central, osmiophilic core (Fig. 2 *c*); organelles resembling melanosomes or Merkel cell granules were never observed.

A large percentage of epidermal TCs reacted with anti-TCR-$\alpha/\beta$ mAbs (Tables I, II). In contrast, TCR-$\gamma/\delta$-bearing cells were seen only occasionally within the basal KC layer and follicular epithelium, as identified by pan $\delta$ chain and anti-V$\delta$1 reagents (Table I), and were most prevalent in the dermis. In contrast to the peripheral blood where almost all TCs express CD7, only 15–30% of the epidermal

$CD2^+/CD3^+$ population were $CD7^+$. As opposed to the situation in peripheral blood (21), $CD8^+$ cells represented the dominant subset within the epidermis (~3:2 ratio of $CD8^+$ to $CD4^+$ TCs), and this pattern was most pronounced in the sole. Although some epidermal TCs were in the activated state, as determined by the expression of HLA-DR, CD25, or late activation antigen VLA-1, the majority lacked these markers (Table II, Fig. 3, *a* and *b*) and were regarded as being in the "resting" state. The sole had fewer activated cells (6% of $CD2^+/CD3^+$ population) than the other body regions (32% of TCs), and in contrast to those in nonvolar sites that were primarily $CD25^+/VLA\text{-}1^-$, activated TCs in the plantar epidermis were $CD25^-/VLA\text{-}1^+$.

To ascertain whether the epidermal TC population consists primarily of "virgin" unsensitized cells or sensitized ones with "memory," we immunolabeled skin sections with the mAbs 2H4 (CD45RA) and UCHL1 (CD45RO), which purportedly identify these reciprocal subsets, respectively (22–25). Most epidermal TCs were primed memory cells (73% of $CD2^+/CD3^+$), based on $2H4^-/UCHL1^+$ reactivity, while only ~20% were $2H4^+$ (Table II). This trend was observed more consistently in the sole than in the trunk and extremities. Efforts to stain with anti-4B4 mAb (CD29), which is specific for the $\beta_1$ chain of the integrin family (reviewed in reference 26) and is reported to identify memory TCs (25), yielded uninterpretable results since the basal KC layer was routinely $4B4^+$, an observation also made by others (7).

Epidermal TCs were randomly distributed between basal/suprabasal KC (Fig. 2) in body regions where the dermal papillae are typically shallow (Fig. 1), with no apparent preference for a particular site within this horizontal plane. However,

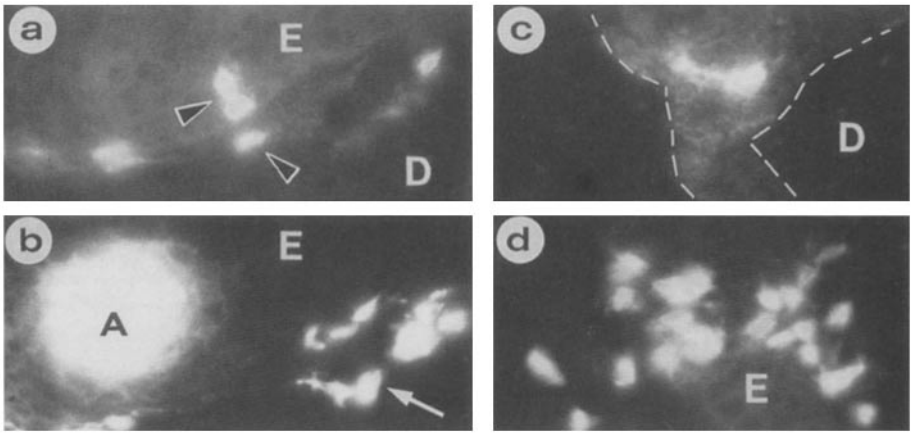


FIGURE 3. Two different double-staining experiments using sole skin. The left side (*a* and *b*) shows one frozen section that was immunolabeled with anti-Leu 4 (*a*, rhodamine stain) and FITC-conjugated anti-HLA-DR (*b*). *a* and *b*, respectively, show $CD3^+/HLA\text{-}DR^-$ epidermal and subepidermal TCs (*arrowheads*) in the same section. Most of the HLA-DR reactivity is restricted to the dermal macrophages, endothelium (*arrow*), and intensely staining acrosyringium (*A*); E, epidermis; D, dermis. ×190. The right panels (*c* and *d*) depict another section of skin that was labeled with FITC-conjugated OKT6 (*c*) and anti-Leu 4 (*d*, rhodamine stain), illustrating the close association between a $CD1a^+/CD3^-$ LC (*c*) within the acrosyringium and numerous epidermal $CD3^+$ TCs (*d*). Dotted line denotes the dermal-epidermal junction, thus showing the position of TCs in the dermis (*D*). ×180.

where the dermal-epidermal contour is more exaggerated (e.g., sole), TCs were more common along the rete ridges than above the dermal papillae and usually appeared to be present in a random, nonclustered pattern, which was most evident by en face examination of epidermal sheets after immunolabeling with a pan-TC marker (Fig. 4 *a*). Epidermal TCs not only had a predilection for rete ridges, particularly around the tip, but also for the outer root sheath of hair follicles and the acrosyringial epithelium of sweat ducts (Fig. 4 *b*). Because of the regular, lattice-like position of sweat glands within the sole and the propensity that TCs apparently have for this epithelium, one could often see a pattern of TC clusters associated with the acrosyringium

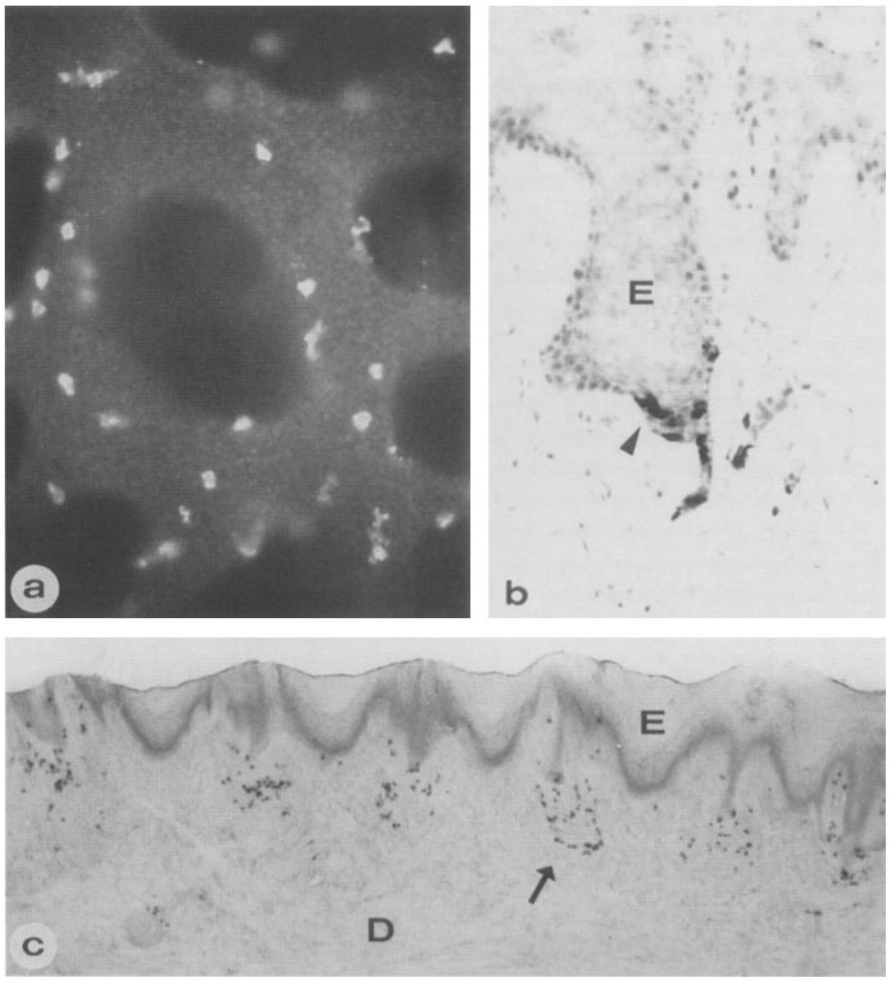


FIGURE 4. Epidermal TCs in plantar skin. (*a*) En face, basal-surface view of an epidermal sheet from the sole showing numerous rhodamine-labeled $CD3^+$ cells distributed along the rete ridges. ×160. (*b*) Frozen section of plantar skin with peroxidase-stained $CD2^+$ cells (*arrowhead*) concentrated within the acrosyringial epithelium of a sweat gland. E, epidermis; ×190. (*c*) Low power micrograph of a thick (>6 μm) frozen section of plantar skin showing epidermal and dermal clusters of peroxidase-labeled $CD8^+$ TCs (*arrow*) associated with the sweat ducts. Note that relatively few $CD8^+$ TCs are present in the reticular dermis (*D*). ×50.

that corresponded to the location of sweat ducts protruding into the dermis through alternating rete ridges (Fig. 4 *c*). Dermal T lymphocytes often were clustered just beneath the basement membrane in these epidermal TC-rich sites, usually in concentric layers two to four cell layers thick. In addition to this focal concentration at the dermal-epidermal junction and within connective tissue sheaths of adnexal appendages, dermal TCs frequently were localized around superficial vessels. However, the concentration of intra- and subepidermal TCs often was strikingly high in comparison to the TC population within the deeper dermis (Figs. 4, *b* and *c*).

There was no statistical difference ($p > 0.01$) in the density of epidermal TCs between thigh, upper arm, buttock, and chest. In these regions, the mean number of $CD2^+/CD3^+$ TC was eight per linear centimeter of epidermis. However, within the epidermis of sole skin (Fig. 4) the density of TCs was significantly higher ($p < 0.0001$), with an average of 40 $CD2^+/CD3^+$ cells per linear centimeter of epidermis (Table II). The density of LCs in the sole was 40% less than in the other four areas examined (Foster, C. A., and B. Volc-Platzer, unpublished data), and because TCs were five times more abundant in the sole epidermis, the ratio of $CD2^+/CD3^+$ TC to LC in plantar skin was 1:1.7 (i.e., 63% of the LC population) as compared with only 1:20 (i.e., 6% of the $CD1a^+$ cells) in other epidermal regions.

*In Situ Relationship of Epidermal TC and LC.* Anti-CD1a/anti-CD3 double labeling revealed that TCs were sometimes in close proximity to LCs in clinically normal-appearing skin (Fig. 3, *c* and *d*). By electron microscopy, it was apparent that this TC-LC association often involved an intimate glycocalyx-to-glycocalyx contact between the two cell types (Figs. 5, 6). In some cases this cellular relationship resembled a tight junction, while in other zones of contact the TC and LC membranes appeared to be fused and were indistinguishable from one another (Fig. 6).

*In Vitro Activation and Characterization of Epidermal T Cells.* When TC-enriched EC suspensions were cultured in 5 μg/ml Con A, 40 U/ml rIL-1, 100 U/ml rIL-2, and 10% A431 cell culture supernatant, a population of club- or Y-shaped cells began proliferating after 4–7 d and finally overgrew the other EC. From 12 different individuals, we established cultures that could be maintained in a growth phase for 4–9 wk and found that the proliferating cells were routinely $CD45^+$, $CD2^+$, $CD3^+$, $CD1a^-$ (FACS profiles not shown). While most of these were usually $CD5^+$, $CD25^+$, and $HLA\text{-}DR^+$, anti-CD7 reactivity was highly variable (10–90%). Although a few cultures consisted of either $CD4^+$ TCs or an equal number of CD4 and CD8 single-positive cells, $CD4^-/CD8^+$ TCs predominated in the majority of cultures, thus closely reflecting the in situ observation. In one culture there was evidence for a substantial $CD4^-/CD8^-$ subpopulation; however, these double-negative cells ceased to proliferate before they could be thoroughly phenotyped.

Immunolabeling for TCR proteins demonstrated that most of the cultures reacted with anti-TCR-$\alpha/\beta$ mAbs (BMA031 and WT31). To investigate the molecular form of TCR-$\alpha/\beta$ dimers in epidermal TCs, some cultures were analyzed by immunoprecipitation using anti-TCR-$\alpha$, anti-TCR-$\beta$, anti-TCR-$\gamma$, and anti-CD3$\delta$ sera. Using digitonin lysates (maintains CD3-TCR bond), the anti-CD3$\delta$ serum precipitated the CD3 complex in association with a 90-kD disulfide-linked heterodimer which, under reducing conditions, dissociated into a 40–45-kD band (Fig. 7 *a*, lane 5). Essentially identical bands of 40–45 kD were precipitated by anti-TCR-$\alpha$ and -$\beta$ but not by anti-$\gamma$ (Fig. 7 *a*, lanes *2–4*) using an NP-40 (dissociates TCR from CD3)

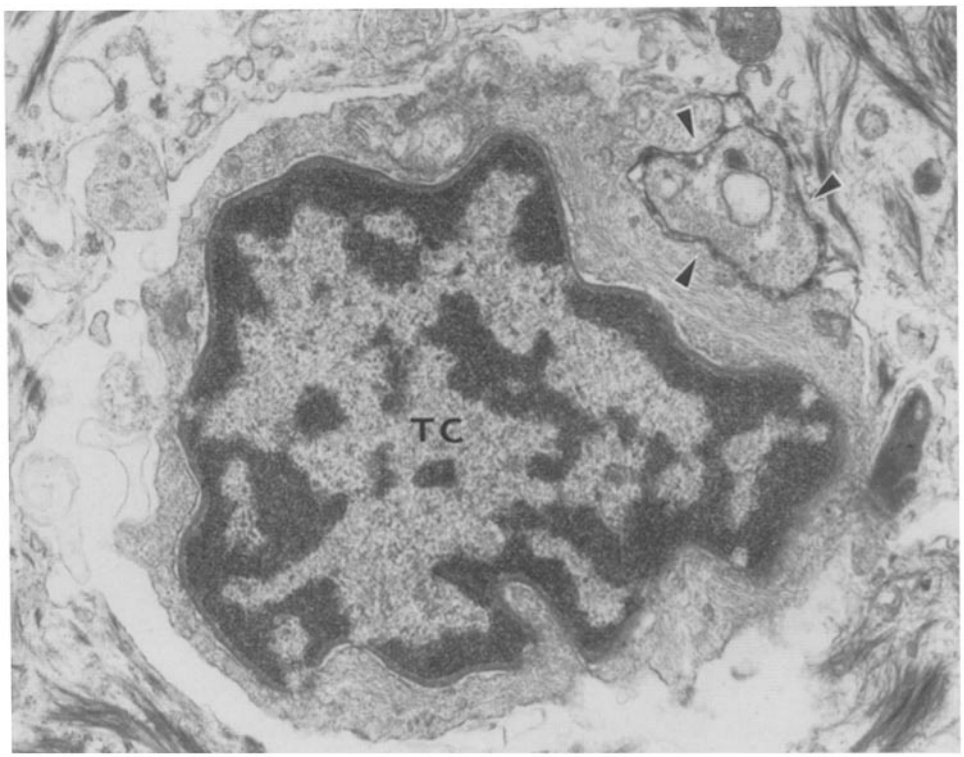


FIGURE 5. Electron micrograph of an epidermal TC (in thigh skin) that is wrapped around a CD1a$^+$ peroxidase-labeled LC process (*arrowheads*). Intermediate size filaments are abundant within the TC cytoplasm. ×16,000.

lysate, indicating that the dimer consisted of disulfide-linked $\alpha/\beta$ chains that appeared identical to those seen in noncutaneous TCs (reviewed in reference 27). In two other epidermal TC cultures (FACS profiles not shown), the cells either were almost exclusively TCR-$\gamma/\delta^+$ (mAbs $\delta$TCS1 and TCR$\delta$1), as well as CD2$^+$/CD3$^+$/CD4$^-$/CD8$^+$ and plastic adherent, or consisted of TCR-$\alpha/\beta^+$ and TCR-$\gamma/\delta^+$ (nonadherent) subpopulations, as demonstrated by immunoprecipitation in Fig. 7 *b*. The protein precipitated with antiserum to TCR-$\gamma$ was ~43 kD (Fig. 7 *b*, lane *4*).

## Discussion

The present study has shown that in clinically normal-appearing adult human skin, epidermal TCs represent a minor but routinely identifiable population. The majority are basally located, CD2$^+$/CD3$^+$/CD5$^+$/CD8$^+$, and exhibit the phenotype of "memory" cells (i.e., 2H4$^-$/UCHL1$^+$) in an unactivated state (i.e., HLA-DR$^-$/CD25$^-$/VLA-1$^-$); more than 50% express the TCR-$\alpha/\beta$ while only a small fraction are TCR-$\gamma/\delta^+$. Epidermal TCs were propagated in vitro from EC suspensions (using Con A, IL-1, IL-2), resulting in the establishment of long-term suspension cultures which by FACS and immunoprecipitation were determined to contain TCR-$\alpha/\beta$- and TCR-$\gamma/\delta$-bearing cells. Furthermore, we demonstrated that epidermal TCs

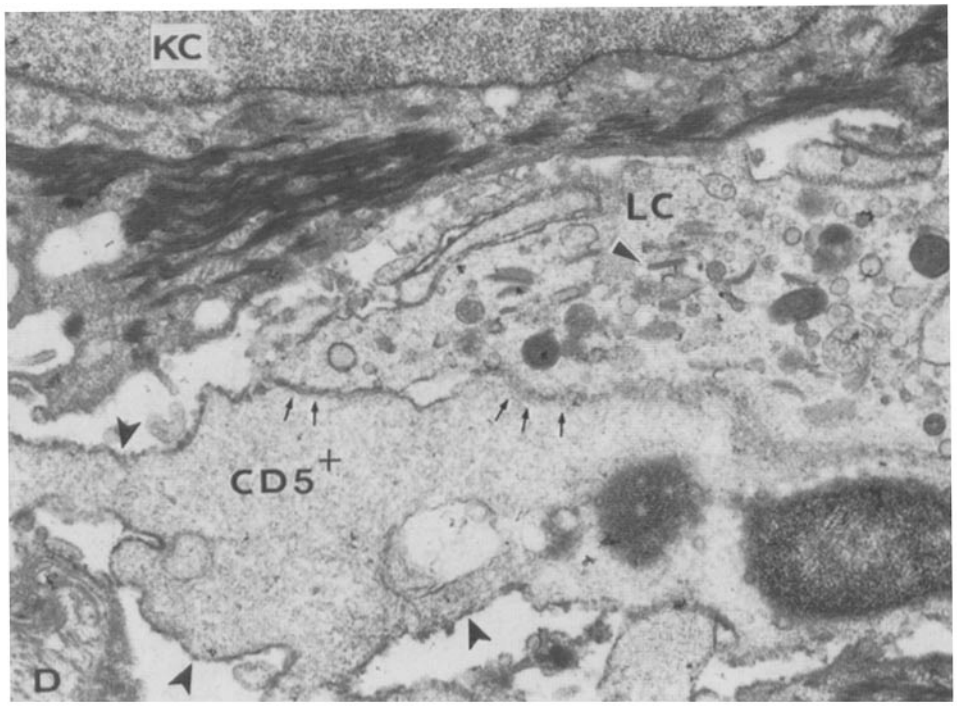


FIGURE 6. Electron micrograph of sole skin illustrating the close association (*small arrows*) between a LC containing Birbeck granules (*small arrowhead*) and an immunolabeled CD5+ epidermal TC located just above the dermis (*D*). The peroxidase-reaction product (*large arrowheads*) is restricted to the TC plasmalemma, which appears to be "fusing" with the LC membrane. KC, keratinocyte. ×18,400.

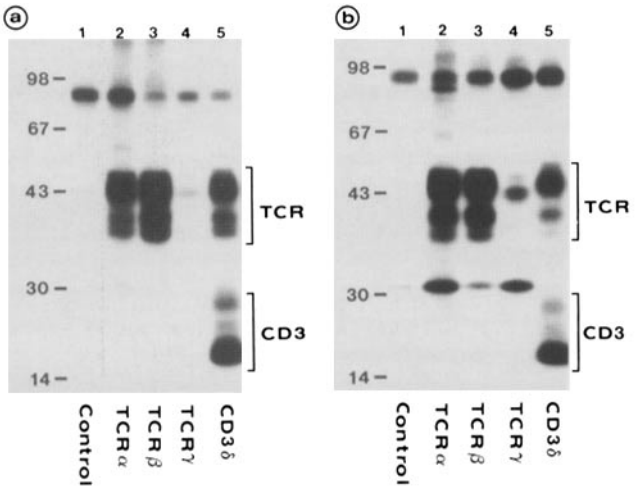


FIGURE 7. Immunoprecipitation and SDS-PAGE analysis of the CD3-associated molecules expressed on two different epidermal TC cultures. The cells used in *a* and *b* were grown in culture (as described in Materials and Methods) for 14 d before $^{125}I$ labeling. Anti-CD3 and anti-TCR immunoprecipitates were made from digitonin and NP-40 lysates, respectively, then analyzed under reducing and nonreducing conditions. Some representative lysates (NP-40, lanes *1–4*; digitonin, lane *5*) under reducing conditions are shown here. Normal rabbit serum (lane *1*); anti-TCR-α (lane *2*); anti-TCR-β (lane *3*); anti-TCR-γ (lane *4*); anti-CD3δ (lane *5*). The positions of the molecular mass markers (kD) are indicated on the left.

were significantly more abundant in the sole than in the trunk and limbs (i.e., 40 vs. 7 $CD3^+$ cells/linear centimeter of epidermis), thus providing the first evidence for significant regional variability within this cutaneous population. Epidermal TCs were often in close association with LCs, and in addition, TC/LC ratios were significantly different in volar vs. nonvolar regions (i.e., $CD3^+/CD2^+$ TCs represent 63% of the number of $CD1a^+$ cells in plantar epidermis as compared with 6% in the trunk and limbs).

Preliminary analyses indicate that palm skin also harbors a larger number of epidermal TCs, as compared with the trunk and limbs (Foster, C. A., unpublished observations). Experiments are in progress to determine which factors are responsible for these observed regional differences, but at the present time we propose the following speculations: (*a*) epidermal TC density is proportional to the concentration and type (eccrine vs. apocrine) of sweat glands within a body area. The enhanced epidermotropism of TCs to plantar skin, which contains the highest number ($620/cm^2$) of eccrine sweat glands (28), may be related to chemoattractants possibly associated with the eccrine ultrafiltrate and/or acrosyringial epithelium (e.g., microbial products gaining access into the skin via adnexal openings, IL-1 [29] and other cytokines, and soluble antigens); (*b*) TC epidermotropism may be enhanced due to altered MHC class II expression by acrosyringial epithelium, the only KC in normal skin that are $HLA\text{-}DR^+$ (30); (*c*) adhesion molecules (reviewed in references 31, 32) expressed by endothelial cells, leukocytes and KC may differ qualitatively and/or quantitatively in certain cutaneous sites; (*d*) the extensive vascular network around sweat glands, coupled with the influence of gravity, may favor increased diapedesis of TCs from subepidermal capillaries and venules to the plantar epidermis. One or more of these conditions could not only selectively attract epidermal TCs but could also discourage the residency of LCs, which are reduced significantly in volar skin (33–35).

We have demonstrated that there are major differences between human and murine epidermal TCs. While the latter cells are uniformly dendritic in shape and express a distinctive TCR-$\gamma/\delta$ phenotype, human epidermal $CD3^+$ cells are variable in shape (spherical to polygonal, or only slightly dendritic), and the vast majority bear TCR-$\alpha/\beta$ rather than $\gamma/\delta$ heterodimers. In contrast to a previous report that >75% of epidermal TCs were $HLA\text{-}DR^+/CD25^+$ and that subepidermal accumulations of TCs were never observed in normal skin (6), the present study demonstrated that only 6–30% expressed markers of activation and that TCs were often more abundant just beneath the basement membrane than throughout the deeper dermis. Furthermore, contrary to the situation in peripheral blood where there are almost twice as many helper/inducer as suppressor/cytotoxic TCs (21) and only about 56% and 40% are $CD45RO^+$, respectively (calculated from references 36 and 37), we observed an approximate 2:3 ratio of epidermal $CD4^+$ to $CD8^+$ TCs, both of which were predominantly $2H4^-/UCHL1^+$. Thus, based on the present study it seems reasonable to speculate that a subpopulation of TCs, previously sensitized to cutaneous antigen(s), specifically "home" to the skin and remain there in a resting state of readiness until coming into contact with the appropriate antigen. This scenario could imply that, at least in the adult, the TCR repertoire of human epidermal TCs is restricted and preselected, rather than promiscuous. Since we have demonstrated that epidermal TCs can be propagated and maintained in long-term culture,

it should be possible to address these issues using molecular biology techniques in conjunction with functional assays.

It is unclear why sometimes only a portion of the $CD2^+/CD3^+/CD5^+$ (TCR-$\gamma/\delta^-$) epidermal cells in frozen sections were labeled by anti-TCR-$\alpha/\beta$ and anti-CD7 mAbs (Table II). One explanation could be limited sensitivity of the immunolabeling technique, but this seems unlikely since the peroxidase reaction product was appropriately intense on those cells that were $\beta F1^+$, $BMA031^+$, or Leu $9^+$. With respect to Leu 9 staining, perhaps CD7 is downregulated/modulated (38) within the epidermal microenvironment or expressed on only a phenotypically unique, but as yet unidentified subset of TCs; interestingly, our epidermal TC cultures also were not uniformly $CD7^+$. Regarding TCR-$\alpha/\beta$, some $CD3^+$ epidermal TCs may only express cytoplasmic CD3 but no surface TCR-associated chains. Finally, it is conceivable that the $CD3^+/TCR\text{-}\alpha/\beta^-/TCR\text{-}\gamma/\delta^-$ cells express a third type of TCR that could not be recognized by our reagents.

At the present time it is technically difficult to clarify the exact physiological role of epidermal TCs, since phenotypic studies have their limitations with regard to biological implications. Our ultrastructural data certainly indicate that LCs can occur in close association with epidermal TCs under normal physiological conditions, forming zones of contact that are similar to those observed in contact dermatitis and mycosis fungoides (39, 40). Thus, the necessary ingredients for an immune response presumably would be available within the epidermis without the necessity for TC recruitment from a regional lymph node and without eliciting signs of an overt immunological reaction. According to our present state of knowledge, this scenario would be most compatible with epidermal $CD4^+$ TCs. However, it is less obvious why $CD8^+$ TCs seem to preferentially migrate into the epidermis, usually as the predominant subset. Do they have tissue-specific homing receptors that are independent of antigen or dependent on prior sensitization? With regard to possible effector functions, are these $CD8^+$ cells MHC-restricted or more similar to natural killer cells; do they secrete a distinct subset of lymphokines? These and other questions remain unanswered until epidermal TC cultures are analyzed for their TCR repertoire and functional activity relative to different cutaneous targets (e.g., allogeneic, autologous, environmental, microbial, and tumor-specific antigens). Such studies should enable us to clarify the postulated role of epidermal TCs in cutaneous immunosurveillance, as defined by the SALT concept (skin-associated lymphoid tissues; 41, 42).

## Summary

The epidermis of clinically normal-appearing human skin harbors a phenotypically heterogeneous population of T lymphocytes (TCs), the majority of which are $CD2^+/CD3^+/CD5^+$ "memory" cells, but in an unactivated state, and express the TCR-$\alpha/\beta$. In contrast to murine skin, only a very minor subpopulation of $CD3^+$ cells in the human epidermis bears the TCR-$\gamma/\delta$. Epidermal TCs primarily are distributed along the rete ridges in the basal keratinocyte layer and are often in close apposition to Langerhans cells (LCs). These TCs were propagated from epidermal cell suspensions after stimulation with TC activating agents (Con A, rIL-1, rIL-2), then evaluated for phenotypic features and TCR diversity. Similar to the in situ situ-

ation, most were $CD4^-/CD8^+/TCR\text{-}\alpha/\beta^+$. In addition, two cultures contained $TCR\text{-}\gamma/\delta^+$ cells; one of these was determined to be an adherent $CD4^-/CD8^+$ population.

Epidermal TCs were significantly ($p < 0.0001$) more abundant in the sole than in the other body regions examined (i.e., 40 vs. 7 $CD3^+$ cells/linear centimeter of epidermis) and seemed to have a particular affinity for the acrosyringial epithelium of eccrine sweat ducts. Moreover, the sole usually contained a greater number of $CD8^+$ relative to $CD4^+$ TCs, whereas the epidermal CD4/CD8 ratio in the trunk and extremities was quite variable, although the trend also was towards a slightly larger percentage of $CD8^+$ cells. Collectively, our data suggest that the volar epidermis has a unique microenvironment which is responsible for both the higher density of TCs, preferentially $CD8^+$, and lower number of LCs. This study has not only provided evidence for significant regional variability in the human epidermal TC population of normal skin, but also strengthens the concept for skin-associated lymphoid tissues (SALT), whereby memory TCs recirculate back to the epidermis and interact with resident antigen-presenting cells (i.e., LC).

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