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# MURINE EPIDERMAL LANGERHANS CELLS MATURE INTO POTENT IMMUNOSTIMULATORY DENDRITIC CELLS IN VITRO

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In addition to keratinocytes, epidermis contains several minor cell populations each of which is "dendritic" in shape. These include Langerhans cells (LC),<sup>1</sup> melanocytes, Merkel cells, and recently described Thy-1<sup>+</sup> cells (1-3). Interest in LC was rekindled several years ago when it became evident that LC might be important in the induction of contact sensitivity (4). Researchers discovered that LC were Ia<sup>+</sup> leukocytes (5-8) that were derived from bone marrow (9), expressed receptors for immune complexes and the third component of complement (C) (Fc and C3 receptors, respectively) (8, 10), and could present antigen to T cells (11-15).

There has been relatively little work on the properties of epidermal LC in tissue culture, and their possible relationship to dendritic cells (DC) isolated from lymphoid organs has not been analyzed. For example, lymphoid DC are active stimulators of T-dependent responses (see 16-21 for examples), yet comparable functional studies on enriched populations of LC have not been done. We will show here that LC come to resemble spleen DC when cultured for 2-3 d. Fresh LC prove to be weak stimulators of T cell proliferation, but undergo an impressive and progressive increase in stimulatory capacity in vitro. We suggest that epidermal LC are precursors or immature elements of the DC system.

## Materials and Methods

*Mice.* C57BL/6, B6.H-2K, B6 × D2 F1, C × D2 F1, and Swiss ICR mice were from the Trudeau Institute, Saranac Lake, NY. B10.BR and B10.T (6R) were from the Mayo Clinic, Rochester, MN. BALB/cAn and Swiss mice were from The Rockefeller University, NY. Mice from both sexes, 6-12 wk of age, gave similar results.

*Epidermal Cell Suspensions.* Epidermal cells were isolated from mouse ear skin by a modification of a previous technique (22), and in the continued presence of gentamicin (20 µg/ml) (Schering Corp., Kenilworth, NJ). Ears were rinsed with 70% alcohol, split with the aid of forceps, and placed, dermal side down, on 7.5 ml Hank's balanced salt solution, without calcium and magnesium (HBSS) (Gibco Laboratories, Grand Island, NY) in 100-mm plastic dishes (3003; Falcon Labware, Oxnard, CA). 5 ml of 2.5% trypsin

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<sup>1</sup> *Abbreviations used in this paper:* BPA, bovine plasma albumin; C, complement; DC, dendritic cell; EM, electron microscopy; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HBSS, Hank's balanced salt solution; HRP, horseradish peroxidase; LC, Langerhans cell; mAb, monoclonal antibody; MLR, mixed leukocyte reaction; PBS, phosphate-buffered saline.

(10 $\times$ ) (Gibco Laboratories) were added to dorsal and ventral ear halves in separate dishes for 20 and 30–35 min, respectively, both at 37°C. Epidermal sheets were peeled from the underlying dermis and floated in 40 ml HBSS with 10% fetal calf serum (FCS) (Sterile Systems, Inc., Logan, UT) in 100-mm dishes, into which large sieves had been placed. The sheets were shaken in the sieve to release cells (primarily cells of the basal epidermal layer) and the sieves were then removed. The cells were vigorously pipetted to break up clumps, then filtered through nylon mesh (NITEX 3-325-44; Tetko, Elmsford, NY). The cells were sedimented at 280 *g* for 10 min at 23°C, and washed twice in RPMI 1640 (Gibco Laboratories) with 10% FCS. In some experiments, dispase (Godo Shusei Co., Matsudo, Chiba, Japan) (500 U/ml HBSS, 30 min at 37°C [23]) was used rather than trypsin. The sheets were peeled off and floated an additional 60 min at 37°C in dispase prior to vigorous pipetting to release cells. The latter were filtered through NITEX mesh and processed as above. Cells that were treated with dispase or trypsin functioned similarly.

Fresh epidermal suspensions were cultured at  $2 \times 10^7$  cells in 10 ml medium (10% FCS in RPMI 1640 with 20  $\mu\text{g}/\text{ml}$  gentamicin) in 100-mm petri dishes (3003, Falcon Labware; or N1415, Nunc, Roskilde, Denmark). After 18–24 h, nonadherent cells were removed by pipetting over the surface several times, and transferred to fresh 100-mm dishes (three dishes of nonadherent cells were pooled into one) for an additional 1–3 d of culture. This transfer left most of the viable keratinocytes adherent to the original dishes, and virtually all of the LC in the nonadherent fraction (see Results). Low and high density fractions of epidermal cells were isolated on bovine plasma albumin (BPA) columns (17, 18) and washed twice with RPMI 1640 prior to use.

Immunofluorescent staining with monoclonal antibodies (mAb) B21-2, anti-Ia<sup>b,d</sup> and 10-2.16, anti-Ia<sup>t</sup> was used to distinguish Ia<sup>+</sup> LC (5–8) from other cell types in epidermal suspensions (1–3). The observed staining was considered specific, since cells from one particular haplotype (H-2<sup>b,d</sup> or H-2<sup>k</sup>) only stained with the corresponding anti-Ia mAb. We will show in the Results that most, if not all, of the isolated Ia<sup>+</sup> cells had the cytologic and antigenic features of LC in situ.

Two techniques were used to deplete Ia<sup>+</sup> cells from epidermal suspensions. For C-mediated lysis, cells at  $2 \times 10^6$  cells/ml were incubated 60 min at 37°C with anti-Ia mAb (B21-2, 10-2.16) and rabbit C in the presence of 30  $\mu\text{g}/\text{ml}$  deoxyribonuclease (Sigma Chemical Co., St. Louis, MO). For panning, 60- or 100-mm bacteriologic petri dishes (1007 or 1005, Falcon Labware) were covered with 40 or 120  $\mu\text{g}$  B21-2 anti-Ia<sup>b,d</sup> Ig at room temperature for 60–90 min. The dishes were washed, and up to 4 or  $10 \times 10^6$  cells were applied for 40–60 min at room temperature. Nonadherent, Ia<sup>-</sup> cells were removed by washing. The adherent cells had cytologic features of LC (not shown), and the recovery of applied epidermal cells in control dishes (no anti-Ia mAb, or B6.H-2k cells applied to anti-Ia<sup>b,d</sup>) was complete.

*Other Cells.* Low density, macrophage-depleted, spleen adherent cells were the source of enriched lymphoid DC (17). Lipopolysaccharide-induced B lymphoblasts and T cells were obtained from spleen (24).

*Electron Microscopy (EM).* Low density epidermal and spleen DC were fixed for 1 h at room temperature in precooled half-strength Karnovsky's paraformaldehyde-glutaraldehyde fixative, and were then processed for EM (25). For immuno-EM, live cells were exposed to anti-Ia antibody (MK-S4, anti-Ia<sup>s</sup>; MK-D6, anti-Ia<sup>d</sup>; American Type Culture Collection, Rockville, MD) diluted in phosphate-buffered saline with 3% bovine plasma albumin (PBS-BPA) and 0.02% sodium azide for 40 min, on ice. After washing, the cells were treated with horseradish peroxidase (HRP)-coupled, F(ab)<sub>2</sub> goat anti-mouse IgG (Tago Inc., Burlingame, CA) diluted 1:10 in PBS-BPA for 40 min, on ice. The cells were washed, fixed as above, then washed in 0.1 M sodium cacodylate, pH 7.4, followed by 0.05 M Tris HCl, pH 7.6, and incubated in hydrogen peroxide-diaminobenzidine (26) for 15 min before further processing for EM.

*Immunofluorescence.* Cells were attached to glass slides coated with poly-L-lysine (50  $\mu\text{g}/\text{ml}$ ) (type VII, Sigma Chemical Co.) and stained, on ice; or the cells were spun ( $200 \mu\text{l}$  of  $3\text{--}5 \times 10^5$  cells/ml in 10% FCS-RPMI 1640) in a Shandon cytocentrifuge (6 min at 800 rpm), followed by air drying. The latter cytospin preparations could be stored in an

airtight box, with dessicant, at  $-20^{\circ}\text{C}$  prior to staining. Smears were fixed with acetone for 5 min at room temperature, washed in PBS, stained at  $20^{\circ}\text{C}$  with the reagents described below, and mounted in PBS-glycerol-azide.

Surface antigens were visualized with hybridoma culture supernatants (see Results) diluted 1:5 in PBS-BPA-azide. In addition, a rabbit anti-keratin reagent was purchased from Dako Corp., Santa Barbara, CA (K518). For mouse mAb, primary antibody was applied for 20 min. The slides were rinsed in PBS and then exposed to affinity-purified F(ab)<sub>2</sub> goat anti-mouse IgG and IgM, fluorescein (FITC)- or rhodamine-conjugate (Grub antibodies, Scandic, Vienna, Austria) at  $20\ \mu\text{g}/\text{ml}$ . For rat mAb, a three-step procedure was used. The mAb was applied for 20 min, followed by a polyclonal mouse anti-rat Ig and rhodamine- or FITC-goat anti-mouse Ig. For double-labeling studies, a blocking solution (rat or mouse Ig at  $250\ \mu\text{g}/\text{ml}$  for 10 min at  $23^{\circ}\text{C}$ ) was added after the first set of reagents. Then, a second antigen was visualized, e.g., FITC-B21-2 anti-Ia<sup>b,d</sup> or -Thy-1.2 (B-D Immunodiagnostics, Orangeburg, NY) or arsenilated anti-Ia<sup>k</sup> and FITC-rabbit anti-arsenilate (B-D Immunodiagnostics).

To identify proliferating cells in culture,  $10\ \mu\text{M}$  bromodeoxyuridine (Sigma Chemical Co.) was included in the culture medium for 15–60 h, followed by fixation and staining with mouse anti-bromodeoxyuridine (30) and FITC-goat anti-mouse Ig, as prescribed by the manufacturer (B-D Immunodiagnostics).

**Cytochemistry.** For assays of membrane ATPase, cytospin preparations were fixed for 1.5 h in 5% formaldehyde in phosphate saline, then washed and stained (31). For staining nonspecific esterase, smears were fixed with buffered formalin-acetone, and stained according to Li et al. (32). To monitor pinocytosis of HRP,  $10^6$  cells were exposed to 1 mg HRP (type II, Sigma Chemical Co.) in 1 ml culture medium for 1 h at  $37^{\circ}\text{C}$ , then washed four times (33). Cytospin preparations were fixed for 5 min in 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, and stained 10 min with diaminobenzidine/ $\text{H}_2\text{O}_2$ .

**Quantitation of Cell Surface Ia Antigens.** At least  $10^5$  cells in 0.7 ml PBS were spun onto 24-well plates (Costar, Rochester Scientific Co., Rochester, NY) coated with poly-L-lysine ( $50\ \mu\text{g}/\text{ml}$ ) (Sigma Chemical Co.). After quenching in HBSS with 5% FCS for 10 min, <sup>125</sup>I-B21-2, anti-Ia<sup>b,d</sup> at  $1\ \mu\text{g}/\text{ml}$ , was added for 60 min, on ice, in  $250\ \mu\text{l}$  HBSS with 5% FCS. The cells were washed, and bound anti-Ia was counted. As described (27, 29), binding was saturable, and haplotype-specific.

**Stimulatory Capacity For T Cell Proliferative Responses.** Graded doses of epidermal or spleen cells (irradiated with 1,500 rad from <sup>137</sup>Cs, unless otherwise indicated) were added to  $3 \times 10^5$  T cells in 96-well plates (Costar 3596, Rochester Scientific Co.). [<sup>3</sup>H]Thymidine uptake was measured, with  $4\ \mu\text{Ci}/\text{ml}$  given at 24–40 h for oxidative mitogenesis (18), and at 72–90 h for MLR (24).

## Results

**The Yield of LC in Fresh and Cultured Epidermal Suspensions.** The yield of epidermal cells was similar in a large number of strains, and averaged  $\sim 2 \times 10^6$  cells per ear,  $\sim 80\%$  of which were viable (Table I). Of these,  $3.0 \pm 1.1\%$  were Ia<sup>+</sup> in C × D2 F1 mice (17 determinations) and 2.5–2.8% in other strains (10 determinations). This corresponds to 800 Ia<sup>+</sup> cells/ $\text{mm}^2$  of skin, which is similar to that observed in intact sheet preparations (34). During 72 h in culture, the number of Ia<sup>+</sup> cells fell progressively to 30% of initial levels (Table II). Yields did not change significantly when suspensions were irradiated (1,500 rad from a <sup>137</sup>Cs source) prior to culture (Table II). That the LC did not proliferate in culture was also shown by the lack of labeling with bromodeoxyuridine, as monitored with a fluorescent antibody (not shown).

**Partial Enrichment of Ia<sup>+</sup> Epidermal LC.** Because LC were a trace component of epidermal cell suspensions, we developed a method for their partial enrichment. Ia<sup>+</sup> LC did not adhere to plastic, whereas most keratinocytes and mela-

TABLE I  
*Epidermal Cell Yields From Ears of Different Mouse Strains*

| Strain            | Cells/ear ( $\times 10^{-6}$ ) | Viable cells |    | Number of experiments |
|-------------------|--------------------------------|--------------|----|-----------------------|
|                   |                                | %            |    |                       |
| B6 $\times$ D2 F1 | 2.1 (0.35)*                    | 81 (3)*      | 6  | 6                     |
| C57B1/6           | 1.5 (0.34)                     | 82 (2)       | 5  | 5                     |
| B6.H-2K           | 1.4 (0.31)                     | 85 (3)       | 10 | 10                    |
| C $\times$ D2 F1  | 1.7 (0.46)                     | 79 (5)       | 26 | 26                    |
| Swiss/NCS         | 2.4 (0.85)                     | 86 (2)       | 5  | 5                     |
| Swiss/ICR         | 1.8 (0.64)                     | 86 (4)       | 2  | 2                     |

\* SD in parentheses.

TABLE II  
*Recovery of Ia<sup>+</sup> Cells (LC) in Epidermal Cultures*

| Exp. | Tissue, treatment                 | Time in culture | Exposure to radiation | Recovery of LC |
|------|-----------------------------------|-----------------|-----------------------|----------------|
|      |                                   |                 |                       | (%)            |
| 1    | Whole epidermis                   | <i>d</i>        |                       |                |
|      |                                   | 0               | —                     | 100            |
|      |                                   | 1               | —                     | 86             |
|      |                                   | 1               | +                     | 96             |
|      |                                   | 2               | —                     | 59             |
|      |                                   | 2               | +                     | 64             |
|      |                                   | 3               | —                     | 34             |
| 2    | Whole epidermis                   | 3               | —                     | 30             |
|      |                                   | 3               | +                     | 39             |
| 3    | Culture 3 d, then float           |                 | —                     | 29             |
|      |                                   |                 | +                     | 26             |
| 4    | Day 0 low density, 3 d in culture |                 | —                     | 19             |
|      |                                   |                 | +                     | 20             |

The number of Ia<sup>+</sup> cells was calculated from: (total cells)  $\times$  (percent Ia<sup>+</sup> profiles in cell smears), and expressed relative to the number of Ia<sup>+</sup> cells at the start of each experiment (100%).

nocytes did. The initial plastic-adherent population contained many round basal keratinocytes and <0.1% Ia<sup>+</sup> cells. By 4 h, the keratinocytes began to spread, and by 3 d, the dishes contained confluent layers of Ia<sup>-</sup> keratinocytes (34).

Flotation on dense albumin columns was used to enrich LC. Flotation at time 0 was partially effective. The low density population contained  $11 \pm 3.8\%$  LC, but only 30% of total LC (Table III). However, when nonadherent or high density cells were cultured one or more days, most of the Ia<sup>+</sup> cells floated on dense albumin. In 2–3 days, the low density fractions were 30–40% Ia<sup>+</sup> (Table III), and expressed several other features of LC (see below). If epidermis was separated into high and low density fractions and then cultured, most of the basal keratinocytes were in the high density fraction. We conclude that cultured

TABLE III  
*Buoyant Density of Fresh and Cultured Epidermal LC*

| Time in culture | Number of experiments | Albumin column fraction | Cell yields<br>( $\times 10^{-6}$ ) | Viable cells<br>% | Ia <sup>+</sup> cells<br>% |
|-----------------|-----------------------|-------------------------|-------------------------------------|-------------------|----------------------------|
| <i>d</i>        |                       |                         |                                     |                   |                            |
| 0               | 16                    | Unfractionated          | 60                                  | 80 ± 5            | 3.1 ± 1.1                  |
|                 |                       | Low density, <1.082     | 5 ± 2                               | >95               | 11 ± 3.8                   |
|                 |                       | High density, >1.082    | 42 ± 11                             | 80 ± 5            | 2.5 ± 0.6                  |
| 3               | 15                    | Unfractionated          | 35 ± 3.5                            | 9 ± 2             | ND                         |
|                 |                       | Low density             | 1.5 ± 0.2                           | >95               | 35 ± 6.4                   |
|                 |                       | High density            | 32 ± 1.5                            | <5                | <0.3                       |
| 2               | 5                     | Low density             | 3.1 ± 0.1                           | >95               | 18 ± 8.8                   |
| 4               | 7                     | Low density             | 0.9 ± 0.2                           | >95               | 45 ± 9.4                   |

Fresh ( $6 \times 10^7$ ) or cultured ( $4 \times 10^7$  nonadherent) mouse epidermal cells were separated into low and high density fractions (BPA,  $P = 1.082$ ) after 0–4 d in culture.

LC are nonadherent, nonproliferating, and low density, while most keratinocytes are adherent, proliferating and high density. Partially enriched, low density nonadherent fractions were used in much of the subsequent work on LC phenotype and function.

*Morphology and Anti-Ia Staining.* By immuno-EM, Ia<sup>+</sup> cells in the starting epidermal suspensions had the known cytologic features (reviewed in 1) of LC, including Birbeck granules (Fig. 1A). A minority of the freshly isolated LC had a much thicker rim of anti-Ia reactivity (compare Fig. 1, A and B). Cultured LC (Fig. 2, A–C) were similar in appearance, except that Birbeck granules were not found, and the cell surface exhibited longer processes. Fresh and cultured LC had many mitochondria but few polysomes, either free or membrane bound. Bundles of intermediate filaments were common, but microtubules were rare. The nucleus was irregular in shape, and the nucleolus was small. The Golgi region had a variable number of electron-lucent, noncoated vesicles. Very few lysosomes and phagosomes with electron-dense contents were seen, even though the LC had been derived from cultures which contained considerable numbers of dead cells and debris.

Light microscopy of cell smears showed that most of the fresh Ia<sup>+</sup> cells were round (Fig. 3, *top*) and lacked the long dendrites that are characteristic of LC *in situ*. A minor fraction (<5%) showed very strong membrane staining, comparable to the findings in immuno-EM (Fig. 1B) and epidermal whole mounts (not shown). The intensity of anti-Ia staining in C × D2 F1 mice was stronger than that observed in other strains (C57BL/6, BALB/c, B6.H-2k, C3H/He). During 3 d of culture, the shape of the Ia<sup>+</sup> cells progressively became less regular, and in cytopsin preparations, long, thin Ia<sup>+</sup> processes were noted (Fig. 3, *middle*). LC could be identified in live cultures, since the processes and/or cytoplasmic veils were evident by inverted phase-contrast microscopy (Fig. 4, *left*).

Cultured spleen DC were compared to cultured LC. The two cell types had similar features by EM (Fig. 2D), immunofluorescence (Fig. 3, *bottom*) and phase-contrast (Fig. 4, *right*).

*Phenotype of LC—Reactivity With mAb.* The reactivity of fresh and cultured

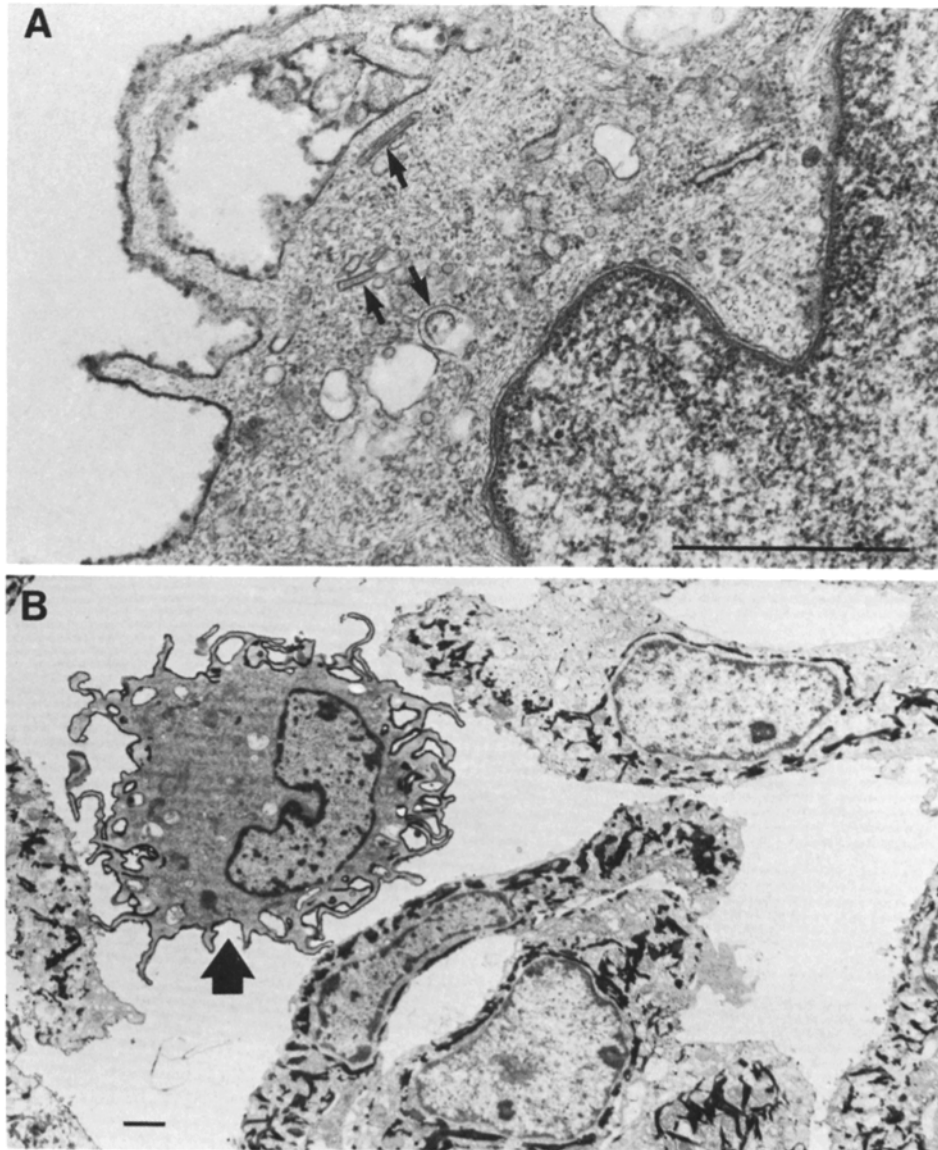


FIGURE 1. Immuno-EM (bar = 1  $\mu$ m) of fresh, low density, epidermal cells stained with anti-Ia mAb. (A) High power showing anti-Ia surface reaction product, Birbeck granules (arrows), and a few lucent, smooth-surfaced vesicles.  $\times 34,000$ . (B) Low power showing several suprabasal keratinocytes and a single LC (arrow) bearing a high level of anti-Ia reaction product.  $\times 5,800$ .

LC with a panel of mAb was studied with a sensitive triple layer technique (Table IV). The phenotype of fresh LC was identical to that observed in intact epidermal sheets (Romani et al., in preparation). In double-staining experiments (Fig. 5), the Ia<sup>+</sup> cells exhibited weak but clear staining with F4/80 anti-macrophage, M1/70 and 1.21J anti-Mac-1 or anti-C3bi receptor, and 2.4G2 anti-Fc receptor, but no staining with anti-T cell (Lyt-1, Lyt-2), anti-B cell (TIB 145 and 146), or

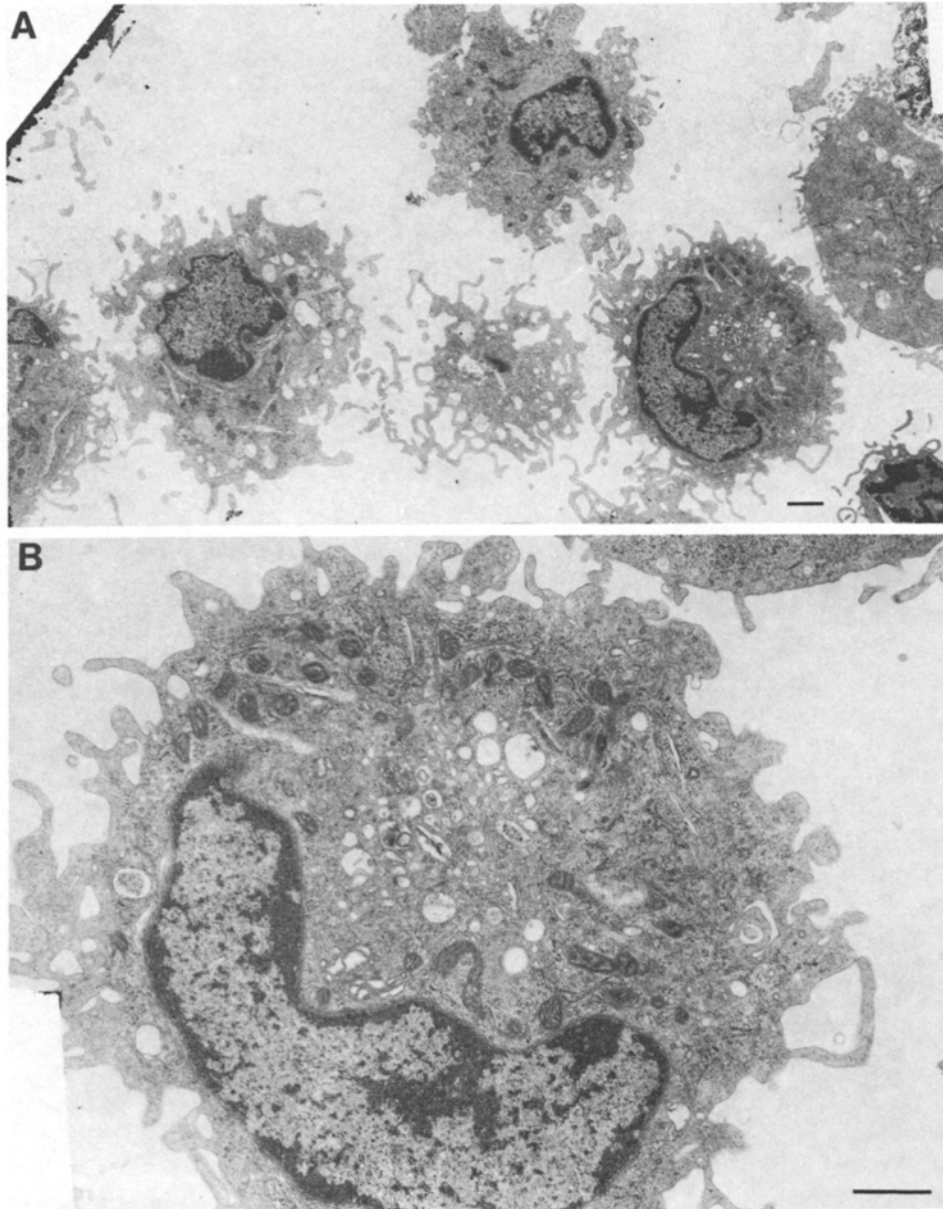
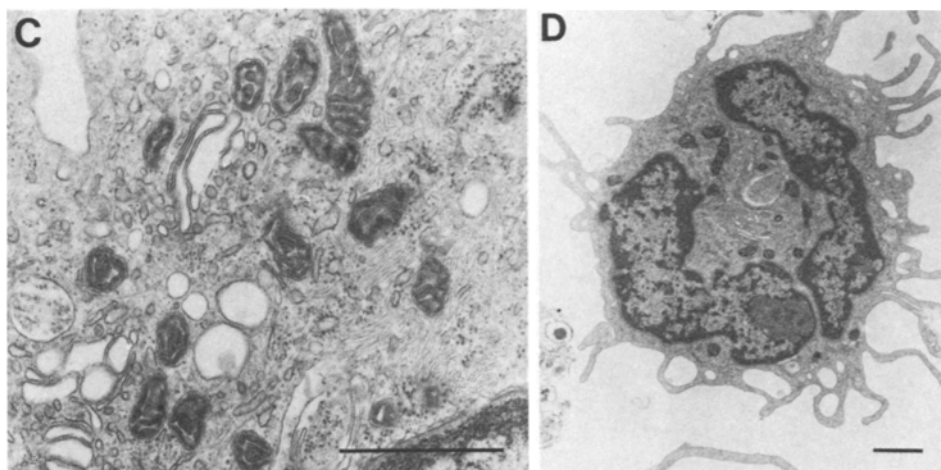


FIGURE 2. EM of 3 d cultured LC (bar = 1  $\mu$ m). (A) Low power to illustrate different surface configurations of LC.  $\times$  4,400. (B) Higher power of one of the LC in A.  $\times$  12,000. (C) High power to show the Golgi and lack of Birbeck granules.  $\times$  25,500. (D) Spleen DC. Note cytologic features similar to LC.  $\times$  7,200.



FIGURE 2. *Continued*

anti-spleen DC (33D1) antibodies. The suspensions contained 0.5–2.0% Thy-1<sup>+</sup> cells, as expected (2, 3), and these lacked LC markers such as Ia and 2.4G2 (Fig. 5).

After 1–3 d in culture, the Ia<sup>+</sup> LC (Fig. 3) could be distinguished from keratin-positive cells (not shown) because of their phase-lucent cytoplasm and irregular cell shape. Anti-Ia staining increased in intensity during culture, but the M1/70 antigen seemed to persist at the initial level (Fig. 5). The 2.4G2 antigen was no longer detectable after overnight culture. F4/80 staining was extremely weak by day 1 and absent by day 2.

The level of Ia on epidermal LC and splenic leukocytes was estimated with binding assays and the <sup>125</sup>I-B21-2 antibody. Fresh LC had 3–4 × 10<sup>5</sup> binding sites per cell, or twice that seen on splenic DC and B lymphoblasts (Table V). The level of Ia increased less than twofold during 3 d of culture.

*Cytochemical Studies of LC and Spleen DC.* LC exhibit nonspecific esterase (36) and membrane ATPase in situ (31, 34) and in fresh suspensions (10, and Romani et al., in preparation). However, both reactivities were not detectable on cultured LC, nor on splenic DC. Cultured peritoneal macrophages were strongly positive (Fig. 6).

We used cytochemistry to study pinocytosis of exogenous HRP (33). Neither cultured LC (Fig. 6) nor spleen DC (not shown) accumulated HRP. In summary, both the antigenic phenotype and cytochemistry of epidermal LC change considerably in vitro and become remarkably similar to spleen DC.

*Stimulatory Capacity For T Cell Proliferative Responses.* It is often stated that epidermal LC are active stimulators of T lymphocyte responses. We were surprised, therefore, to find that fresh epidermal cell suspensions were relatively weak stimulators (Figs. 7, 8; Table VI) of the mixed leukocyte reaction (MLR) and the polyclonal mitogenesis of periodate-modified T cells (oxidative mitogenesis). However, when epidermal cells were maintained in culture, marked stimulatory activity developed progressively over a 3 d period (Fig. 7). Total stimulatory activity of the cultured suspension typically increased at least 10-fold,

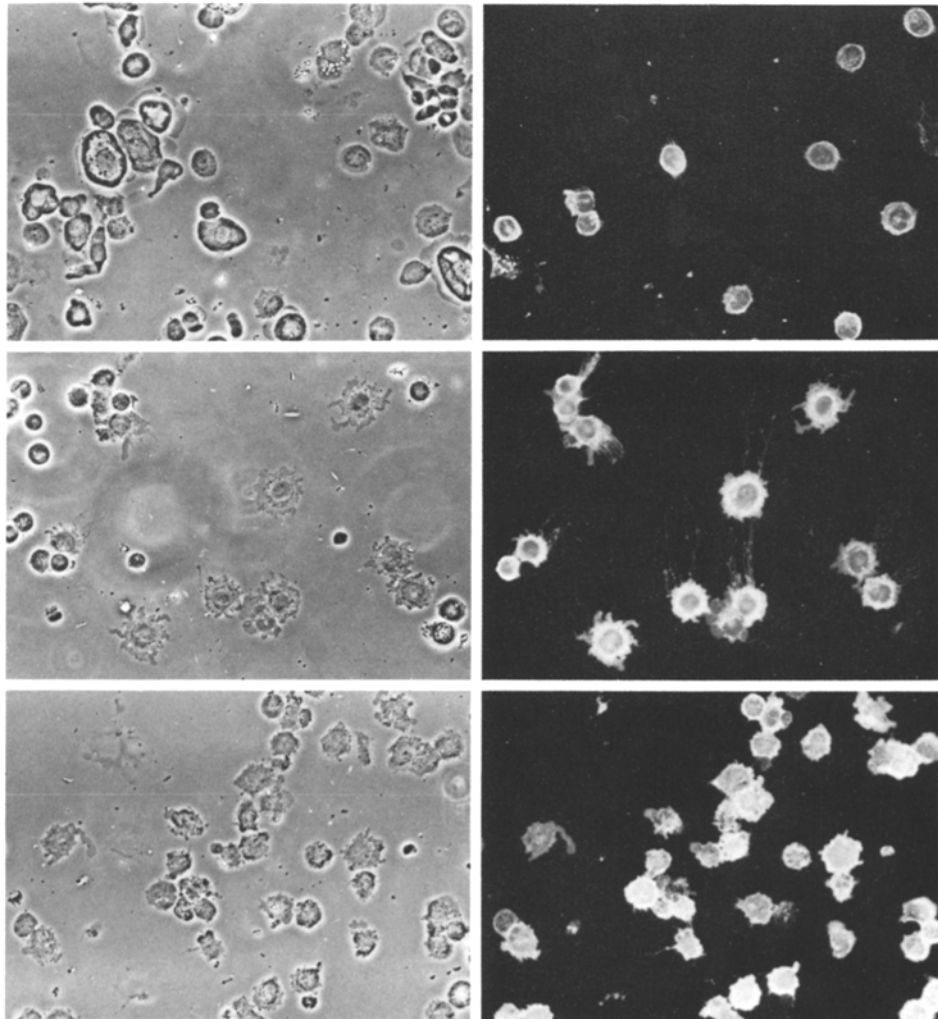


FIGURE 3. Cytology (phase-contrast of cell smears, *left*) and expression of Ia antigens (immunofluorescence, *right*). See text for details.  $\times 400$ .

and the activity per Ia<sup>+</sup> cell increased at least 30-fold. Similar findings were made when we studied whole epidermal cell cultures (Fig. 7), low density, LC-enriched populations prepared after varying times in culture (Table VI), or low density populations isolated at day 0 and cultured 3 d (Fig. 8). Epidermal cells that were irradiated (1,500 rad from a <sup>137</sup>Cs source) prior to culture for 2–3 d (Fig. 7), or prior to coculture with T cells (Table VI) were unaltered in stimulatory activity. Addition of indomethacin, to block the potential immunosuppressive effects of epidermal prostaglandins, did not alter stimulatory function (Fig. 8).

As a result of this “maturation” in culture, LC became 3–10-fold more immunostimulatory than spleen DC (Table VI). 100 cultured epidermal cells, containing ~30 Ia<sup>+</sup> LC, induced marked proliferative responses (Fig. 8). In all cases where epidermal cells induced T cell proliferation, we noted the develop-

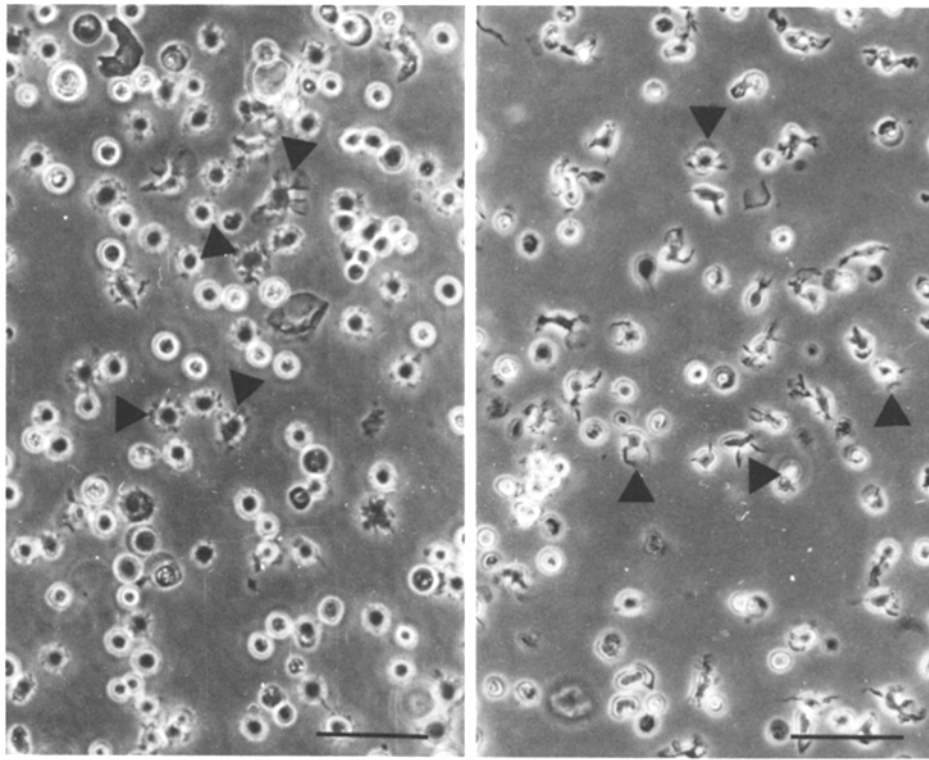


FIGURE 4. Phase-contrast of cultured epidermis, low density fraction (*left*) and spleen DC (*right*). Note the high frequency of profiles with cytoplasmic processes or veils (arrowheads). (bar = 100  $\mu$ m).  $\times$  150.

TABLE IV  
*Antigens of Epidermal LC and Spleen DC*

| mAb            | Directed against | LC    |         |       |       | DC (day 1)     |
|----------------|------------------|-------|---------|-------|-------|----------------|
|                |                  | day 0 | day 1   | day 2 | day 3 |                |
| B21-2, 10-2.16 | Ia               | +     | +       | +     | +     | +              |
| F4/80          | Macrophage       | +     | $\pm^*$ | —     | —     | — <sup>‡</sup> |
| 2.4G2          | Fc receptor      | +     | —       | —     | —     | —              |
| M1/70          | C3biR            | +     | +       | +     | +     | + <sup>§</sup> |
| 33D1           | Dendritic cell   | —     | —       | —     | —     | +              |
| B5-3           | Thy-1.2          | —     | —       | —     | —     | —              |
| 53-7.3         | Lyt-1            | —     | —       | —     | —     | —              |
| 53-6.7         | Lyt-2            | —     | —       | —     | —     | — <sup>‡</sup> |

A panel of mAb (27, 29) was used to stain smears of epidermal cells (cultured 0–3 d) and enriched populations of spleen DC. Only the anti-Ia reagents showed strong immunofluorescence. The determinations have been made at least five times, including at least two experiments with double staining (using anti-Ia and another mAb; Fig. 5).

\* All LC were stained, but the staining was very weak.

<sup>‡</sup> A small fraction (<10%) of the DC-enriched population was stained.

<sup>§</sup> Mac-1 was not previously detected on DC (27).

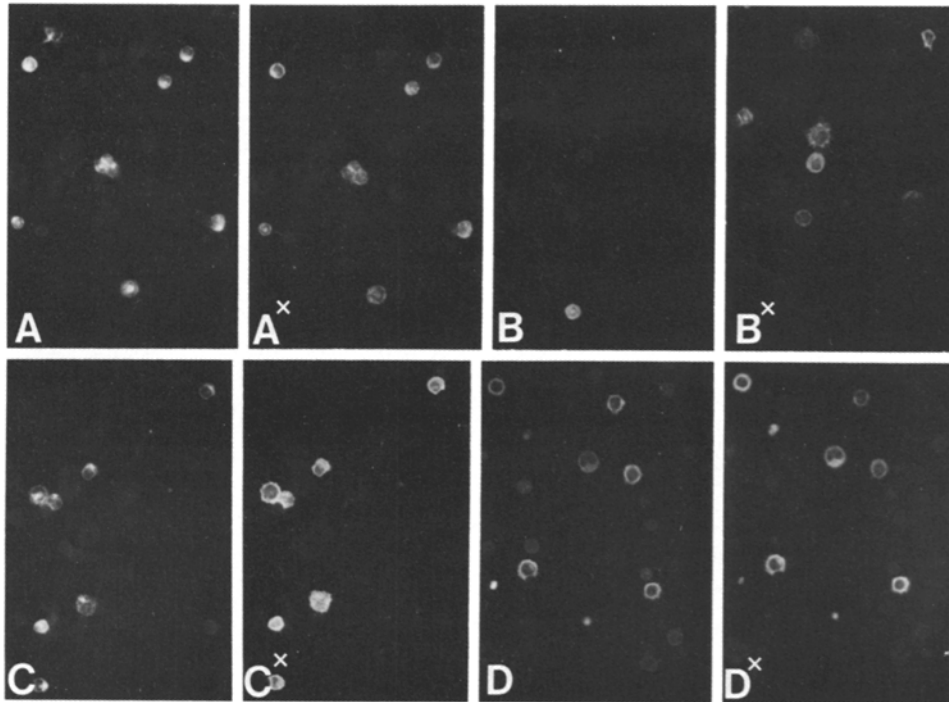


FIGURE 5. Double-staining of low density epidermal cells with pairs of mAb. The anti-Ia and anti-Thy-1 were direct stains with FITC-conjugated mAb, while the 2.4G2 and Mac-1 staining required a triple-layer technique. Each field had a total of ~20 cell profiles, but the keratinocytes did not stain with the mAb.  $\times 180$ . (A, A\*) Fresh epidermis stained with anti-Ia (left) and 2.4G2 (right). (B, B\*) Fresh epidermis stained with anti-Thy-1 (left) and 2.4G2 (right). (C, C\*) Fresh epidermis stained with anti-Ia (left) and anti-Mac-1 (right). (D, D\*) 2-d cultured epidermis stained with anti-Ia (left) and anti-Mac-1 (right).

ment of large cell aggregates, comparable to those seen in responses induced by spleen DC (17, 24, 45).

To verify that  $Ia^+$  cells were essential for stimulatory function, we showed that treatment with anti-Ia plus C (Table VII), or panning on anti-Ia-coated dishes (see Table X below) totally removed activity. Treatment with anti-DC antibody 33D1 plus C (16) did not reduce stimulation. If  $Ia^+$  LC were depleted by panning at the onset of culture, neither  $Ia^+$  cells nor stimulatory function developed (Fig. 9).

Several experiments were then performed to verify that fresh epidermal LC were not inhibited, and that cultured cells did not contain some amplifier of stimulator function. Exposure of cultured epidermis or spleen DC to the enzymes needed to dissociate skin (trypsin or dispase) did not reduce stimulatory function (Table VIII). When day 0 epidermal suspensions were mixed with cultured LC or spleen DC, no inhibition was noted except at very high doses (Table IX). This inhibition was reversed by the inclusion of indomethacin in the cultures (Table IX). Addition of epidermal cell-conditioned medium did not enhance the stimulatory function of fresh epidermal cells (not shown), nor did addition of cultured

TABLE V  
Quantitation of Ia Antigens on LC and Other Leukocytes

| Mouse strain      | Cells analyzed | Time in culture | Number of experiments | Anti-Ia binding sites per cell ( $\times 10^{-5}$ ) |
|-------------------|----------------|-----------------|-----------------------|---|
| C $\times$ D2 F1  | Epidermal LC   | <i>d</i>        |                       |   |
|                   |                | 0.2             | 3                     | 323 $\pm$ 45  |
|                   |                | 2               | 1                     | 530   |
|                   |                | 0.75            | 4                     | 471 $\pm$ 41  |
| B6 $\times$ D2 F1 | Epidermal LC   | 5               | 1                     | 390   |
|                   |                | 2               | 1                     | 450   |
| BALB/c            | Epidermal LC   | 4               | 1                     | 406   |
|                   |                | 3               | 1                     | 612   |
| C $\times$ D2 F1  | B lymphoblasts | 2               | 2                     | 130 $\pm$ 35  |
| C $\times$ D2 F1  | Spleen DC      | 1               | 2                     | 165 $\pm$ 45  |
| BALB/c            | Spleen DC      | 1               | 1                     | 276   |

The level of Ia on different cell populations was measured using a binding assay with  $^{125}\text{I}$ -B21-2. The level of bound antibody was  $15\text{--}40 \times 10^3$  cpm/well. Binding to Ia<sup>k</sup> cells was  $<200$  cpm.

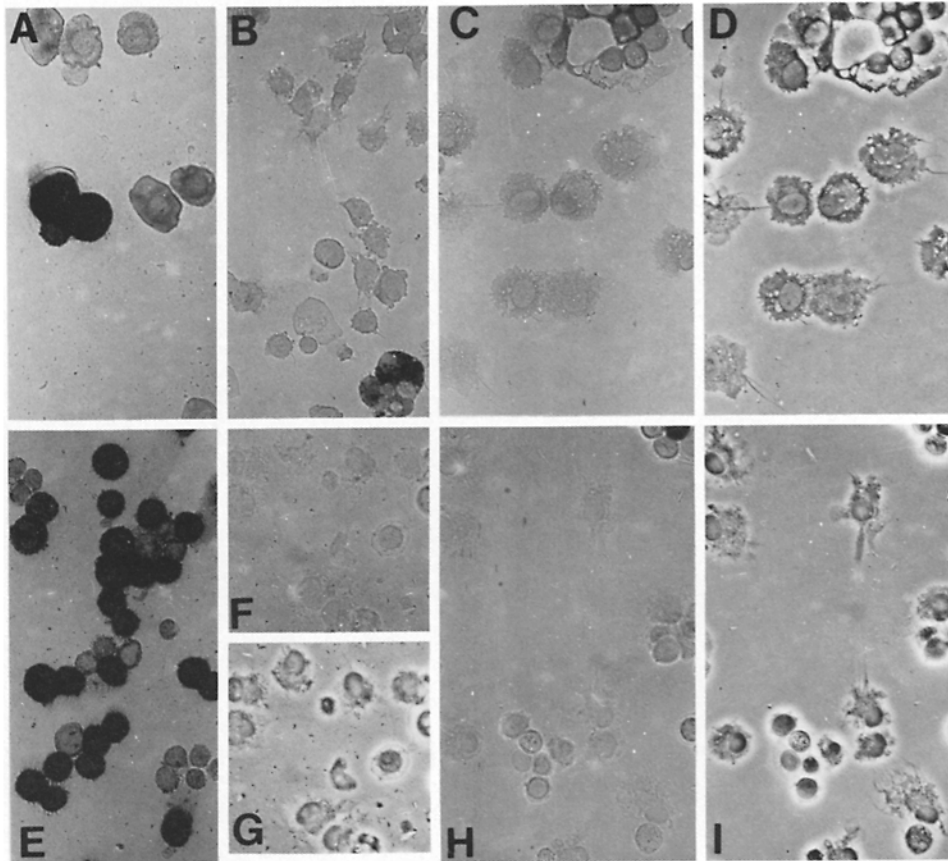
Ia<sup>-</sup> cells (Table X). We conclude that Ia<sup>+</sup> LC progressively mature into active stimulator cells during 3 d of culture.

### Discussion

Lymphoid organs contain Ia<sup>+</sup> DC which are specialized accessory or stimulator elements for T-dependent immune responses (16–20). DC are derived from the bone marrow (37, 38), but few details are known about their development from less mature precursors, or their possible relationship to Ia<sup>+</sup> DC in tissues (39, 40). Ia<sup>+</sup> epidermal LC (reviewed in 1) may provide insight into these unknowns.

*Resemblance of Cultured LC to Spleen DC.* Both cell types are virtually indistinguishable by morphologic criteria (Figs. 2–4). Both are nonadherent, have a low buoyant density (Table III), express large amounts of Ia antigens and small amounts of C3bi receptor or “Mac-1” (Fig. 5, Tables IV, V), and fail to accumulate the pinocytic tracer HRP (Fig. 6). A number of features of freshly isolated LC, which are not found on spleen DC, are lost during culture; e.g., Birbeck granules (Fig. 2), F4/80 and 2.4G2 antigens (41, 42, and Table IV), and cytochemical activities like nonspecific esterase and membrane ATPase (31, 34, 36, and Fig. 6). Fresh LC are weak stimulators of primary T cell proliferative responses (MLR and oxidative mitogenesis), whereas fresh spleen DC are very active (16, 19). However, during culture, LC become more active than spleen DC (Tables VI–X and Figs. 7 and 8). The LC is, in fact, the most potent immunostimulatory element yet identified. As few as 30 cells induce significant responses in standard cultures of  $3 \times 10^5$  T lymphocytes (Fig. 8).

Some differences between LC and lymphoid DC exist. The 33D1 trypsin-



**FIGURE 6.** Cytochemical studies.  $\times 180$ . (A) Nonspecific esterase, fresh low density epidermal cells, bright-field.  $<10\%$  of the cells stain strongly, but many others stain above background. Because fresh LC have regular cell shapes, we could not tell if the positive cells included LC. (B) Nonspecific esterase, spleen DC, bright-field. All cells are negative except for a single contaminating macrophage. (C and D) Nonspecific esterase, 3-d cultured epidermal cells; low density fraction; bright-field and phase-contrast. The LC are nonreactive. (E) Membrane ATPase, peritoneal cells, bright-field. The large macrophages are strongly positive, and served as a positive control. (F and G) Membrane ATPase, 3-d cultured epidermal cells; low density fraction, bright-field and phase-contrast. The LC are nonreactive. (H and I) Peroxidase, d-3 low density epidermal cells, bright-field and phase-contrast. The cells were exposed to 1 mg/ml HRP for 1 h prior to washing and fixation. Accumulation of HRP is not seen, except for an occasional round cell (top right corner).

resistant antigen is found on DC in Peyer's patch, spleen, and node (16, 43, 44) but not on LC (Tables IV and VII). Also, the survival of LC in vitro is not affected by exposure to irradiation (Table II and Fig. 7), whereas most splenic DC do not survive more than a day after such treatment (our unpublished observations). These differences may reflect an influence of keratinocytes.

*Relationship of LC to Mononuclear Phagocytes.* The presence of the macrophage-restricted F4/80 antigen on fresh LC points to a relationship between LC and mononuclear phagocytes, as has been suggested by Hume et al. (41). Nevertheless, several features of LC are atypical for macrophages. LC lose all

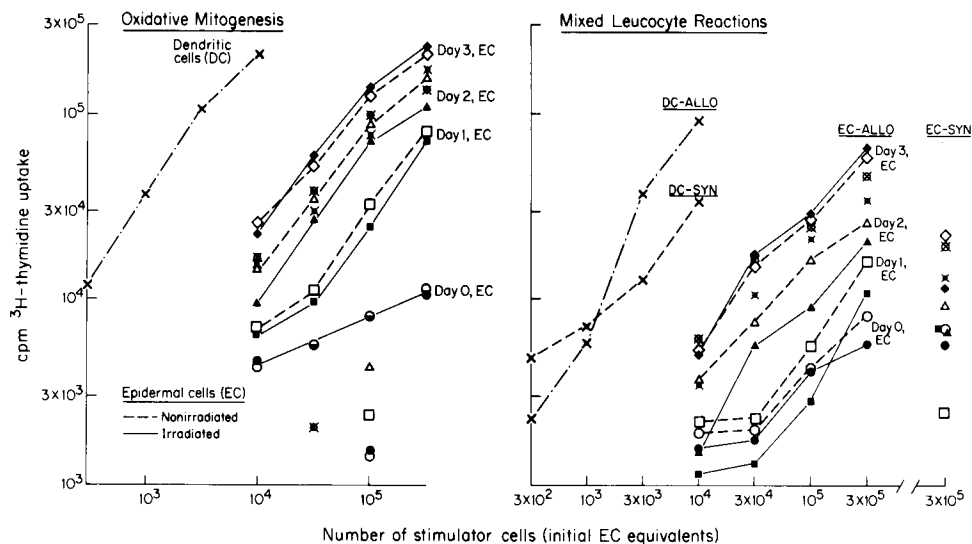


FIGURE 7. The maturation of epidermal stimulatory function for T cell proliferation. On days 0–4, C × D2 F1 epidermal suspensions, with or without 1,500 rad irradiation, were either plated in microwells in graded doses, or in 35-mm-diam cells. For the latter, nonadherent cells (depleted of most viable keratinocytes) were harvested, washed, brought to the original volume, and used to determine the number of Ia<sup>+</sup> cells (see Table II, exp. 1) or as stimulators (data are shown in the figure and were similar to those not presented here for whole epidermal cultures). After the epidermal cells had been cultured 0, 1, 2, 3, and 4 d (■, □, ●, ○), syngeneic C × D2 F1 or allogenic C3H T cells were added, and proliferative responses were measured at 24–40 h (oxidative mitogenesis) or 72–90 h (MLR).

detectable F4/80 and 2.4G2 antigens in culture, exhibit little bulk endocytosis of particles and soluble tracers, fail to adhere to glass or plastic, and give rise to lymphoid-type DC in culture. Therefore, LC represent a distinct leukocyte differentiation pathway, perhaps influenced by the epidermis, and perhaps typical of DC in other nonlymphoid tissues (see below).

*Evidence That Epidermal LC Are Immunologically Immature.* We think that the changes that occur in culture reflect the maturation of individual LC from an inactive to an immunologically active state. We were concerned that the process of epidermal dissociation had altered LC function. Yet our suspensions were viable (Table I), normal in ultrastructure (Fig. 1), and expressed substantial amounts of Ia (Table V). The trypsin used for epidermal dissociation did not inhibit stimulatory function (Table VIII), and contaminating Ia<sup>-</sup> epidermal cells were not inhibitory (Tables IX and X). The idea that LC were differentiating in culture is supported by the selectivity of the changes we observed. Some LC features were lost (see above), some unchanged (expression of Mac-1 and fine structure), and some enhanced (Ia expression, cell size, and function).

The maturation of LC function probably cannot be ascribed to an increase in the level of Ia. Quantitative binding studies revealed that the starting amount of Ia was already greater than spleen DC, and increased by no more than twofold in culture (Table V). Of particular interest was the fact that cultured LC, like spleen DC (17, 24, 45), formed large clusters with responding T cells, whereas

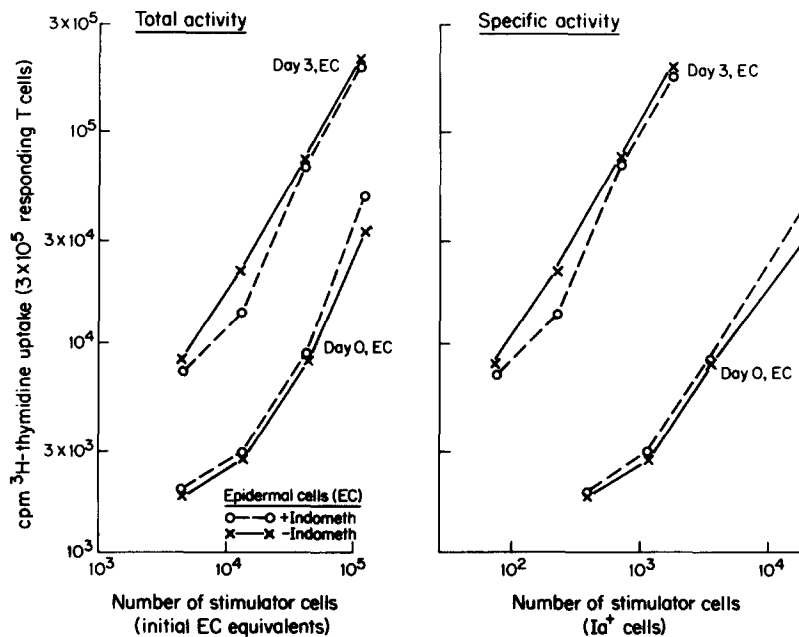


FIGURE 8. Maturation of LC function in cultured, low density cells. C × D2 F1 low density cells were used to stimulate periodate-modified nylon T cells immediately after flotation (day 0) or after 3 d of culture. Stimulator doses are given in terms of Ia<sup>+</sup> cells (specific activity) or initial epidermal equivalents (total activity). The latter means that the starting cell suspension was brought to  $1.2 \times 10^6$  viable cells/ml, and 0.1 ml was the highest dose. A replicate suspension was cultured for 3 d, washed, brought to the original volume, and the same 0.1 ml maximum dose was used (even though the viable cell recovery was 20%). Two populations of day 0 cells were studied with identical results. One day 0 preparation was made at the onset of the 3-d culture, while another was made at the time the 3-d cultures were ready to be tested. For day 3 cultures, we tested cells that had been maintained in graded doses in microtest wells for 3 d prior to addition of T cells (shown here); or we cultured for 3 d in petri dishes, removed the nonadherent cells, washed, and brought the suspension to the original volume (not shown, but the results were identical).

fresh LC did not. The capacity to cluster may have allowed the cultured LC to become competent in T cell stimulation.

We suspect that some of the events described in culture begin within the epidermis *in situ*. Romani et al. (in preparation) note that epidermal sheets contain a small (<5%) subpopulation of LC that stains more strongly with anti-Ia, and therefore resembles cultured LC. Perhaps LC begin to mature in skin and then leave via the lymph. There is evidence that afferent lymph in most species contains a sizable number of cells that resemble LC and lymphoid DC in morphology and phenotype (39, 46–49). Functional data indicate that these “veiled” afferent lymph cells stimulate T cell proliferative responses (39, 49), and therefore may represent the *in situ* analogue of the cultured LC.

Although the literature has not emphasized this point, there do not seem to be instances in which lymphoid suspensions develop such enhanced stimulatory activity during culture, as occurs with cultured epidermis. For example, we have not seen a marked increase in total stimulatory function in cultured human tonsil or blood, or in mouse spleen (our unpublished observations). Therefore, nonlym-



TABLE VI  
*Epidermal Cells and Splenic DC Stimulation of T Cell Proliferation ( $^3\text{H}$ Thymidine Uptake)*

| Stimulators                        | Irradiation | Oxidative mitogenesis |                 |                 | Allogeneic MLR  |                 |                 | Syngeneic MLR   |
|------------------------------------|-------------|-----------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                    |             | $2 \times 10^4$       | $7 \times 10^5$ | $2 \times 10^5$ | $2 \times 10^4$ | $7 \times 10^5$ | $2 \times 10^5$ | $2 \times 10^4$ |
| <i>cpm</i> $\times 10^{-3}$        |             |                       |                 |                 |                 |                 |                 |                 |
| Day 0 epidermal cells, low density | -           | 4                     | 2               | 2               | 0.6             | 0.4             | 0.6             | 0.3             |
|                                    | +           | 8                     | 3               | 2               | 1               | 1               | 0.6             | 0.3             |
| Day 2 epidermal cells, low density | -           | 188                   | 142             | 82              | 197             | 124             | 65              | 23              |
|                                    | +           | 174                   | 128             | 43              | 176             | 108             | 45              | 16              |
| <i>cpm</i> $\times 10^{-3}$        |             |                       |                 |                 |                 |                 |                 |                 |
|                                    |             | $1 \times 10^4$       | $3 \times 10^5$ | $1 \times 10^5$ | $1 \times 10^4$ | $3 \times 10^5$ | $1 \times 10^5$ | $1 \times 10^4$ |
| Day 0 spleen DC                    | -           | 126                   | 74              | 29              | 102             | 45              | 13              | 2               |
|                                    | +           | 116                   | 70              | 31              | 89              | 33              | 7               | 0.8             |
| Day 2 spleen DC                    | -           | 136                   | 68              | 16              | 114             | 33              | 6               | 2               |
|                                    | +           | 117                   | 44              | 11              | 95              | 23              | 3               | 2               |
| Nylon T cells only                 |             |                       | 2               |                 |                 | 0.6             |                 | 0.4             |

Four populations of B10.BR (H-2<sup>bkt</sup>) cells were tested as stimulators for three T cell proliferative responses. Day 0 low density epidermal cells were 7.5% Ia<sup>+</sup>, while day 2 low density cells were 24% Ia<sup>+</sup>. Stimulators were tested with and without exposure to 1,500 rad ionizing radiation, given at the time the T cells were added. Responding T cells were B10.BR for oxidative mitogenesis and syngeneic MLR, and B10.T (6R) (H-2<sup>qrd</sup>) for the allogeneic MLR.

TABLE VII  
*Treatment With Anti-Ia Plus C Ablates LC Stimulatory Function*

| Stimulator cells  | Treated with C plus: | $^3\text{H}$ Thymidine uptake with stimulator doses of: |                 |                 |                 |
|---|----------------------|---|-----------------|-----------------|-----------------|
|   |                      | $9 \times 10^4$   | $3 \times 10^4$ | $1 \times 10^4$ | $3 \times 10^3$ |
| <i>cpm</i> $\times 10^{-3}$   |                      |   |                 |                 |                 |
| Day 2 and 3 C $\times$ D2 F1, low density fraction (15% Ia <sup>+</sup> ) | alone                | 55  | 29              | 10              | 7               |
|   | 33D1                 | 62  | 36              | 10              | 4               |
|   | anti-Thy-1           | 61  | 29              | 11              | 4               |
|   | anti-Ia              | 0.3   | 0.3             | 0.3             | —               |
| Day 4 Swiss mouse cells, low density fraction (36% Ia <sup>+</sup> )      | alone                | 110   | 42              | 15              | 4               |
|   | 33D1                 | 97  | 34              | 10              | 2               |
|   | anti-Ia              | 0.5   | 0.3             | 0.5             | 0.4             |

Cultured epidermal cells, low density fractions, were treated with mAb and rabbit C, washed, irradiated, and used in graded doses to stimulate periodate-modified T cells (background of 1,460 and 288 cpm).

phoid organs like epidermis may be the site where immature accessory cells are located. The yield of LC from two ears (~5% of total skin area) is equivalent to the yield of spleen DC. Some of the dendritic Ia<sup>+</sup> leukocytes that have been found in other nonlymphoid organs (39, 40) also may resemble LC, and may ultimately mature and migrate, via the afferent lymph, to lymph nodes to provide competent accessory cells for the induction of T-dependent immune responses.

### Summary

Murine epidermal Langerhans cells (LC) have been studied in tissue culture and compared to spleen dendritic cells (DC). LC comprised 3% of the starting

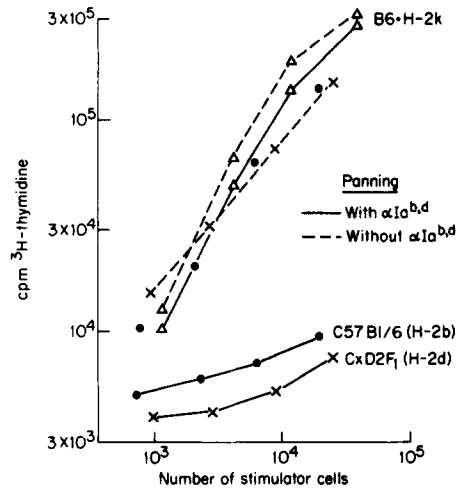


FIGURE 9. Application of fresh epidermal cells to anti-Ia-coated dishes (panning) removes Ia<sup>+</sup> cells and stimulating activity. After panning, the nonadherent cells (>80% of the applied number in all cases) were cultured 3 d, and the low density fractions were used as stimulators of oxidative mitogenesis. Aliquots of all three suspensions were stained with anti-Ia to prove that Ia<sup>+</sup> cells had been depleted and did not develop in culture.

TABLE VIII

*Treatment With Neither Trypsin Nor Dispase Reduces Stimulatory Function of Skin LC and Spleen DC*

| Stimulators                        | Treatment | Ia <sup>+</sup> | [ <sup>3</sup> H]Thymidine uptake with stimulator doses of: |                       |                       |                       |
|------------------------------------|-----------|-----------------|---|-----------------------|-----------------------|-----------------------|
|                                    |           |                 | 7 × 10 <sup>4</sup>   | 2.3 × 10 <sup>4</sup> | 7.8 × 10 <sup>3</sup> | 2.6 × 10 <sup>3</sup> |
|                                    |           | %               | <i>cpm × 10<sup>-5</sup></i>                                |                       |                       |                       |
| Low density epidermal cells, day 2 | None      | 35              | 153   | 143                   | 114                   | 103                   |
|                                    | Trypsin   | 32              | 299   | 269                   | 185                   | 135                   |
|                                    | Dispase   | 33              | 238   | 202                   | 170                   | 106                   |
|                                    |           |                 | 3 × 10 <sup>4</sup>   | 1 × 10 <sup>4</sup>   | 3 × 10 <sup>3</sup>   | 1 × 10 <sup>3</sup>   |
|                                    |           |                 | <i>cpm × 10<sup>-5</sup></i>                                |                       |                       |                       |
| Spleen DC                          | None      | >90             | 116   | 50                    | 10                    | 3                     |
|                                    | Trypsin   |                 | 108   | 43                    | 10                    | 3                     |

Cultured epidermal cells (low density fractions) and spleen DC were treated at 10<sup>6</sup> cells/ml in HBSS and 0.025% deoxyribonuclease with either 0.25% trypsin (15 min, 37°C) or dispase (500 U/ml for 60 min at 37°C) prior to use as stimulators of oxidative mitogenesis. Uptake for T cells only was 1,464 cpm. The "enhancing" effect of protease treatment of day 2 epidermal cells was not observed in two subsequent experiments.

cell suspensions and were distinguished from keratinocytes by cytology and reactivity with anti-Ia and anti-Mac-1 monoclonal antibodies. The LC were nonadherent, had a low buoyant density, did not proliferate, and could be enriched to 10–50% purity. LC continued to exhibit Ia and Mac-1 antigens for 4 d in culture. However, LC rapidly lost Birbeck granules, Fc receptors, F4/80 antigen, and cytochemical reactivity for nonspecific esterase and membrane

TABLE IX  
*Fresh Epidermal Cells Do Not Inhibit Stimulatory Activity For T Cell Proliferation*

| Stimulator cells  | Indo-<br>methacin<br>(1 $\mu$ g/ml) | [ <sup>3</sup> H]Thymidine uptake with stimulator<br>doses of: |                                 |                               |                                 |
|---|-------------------------------------|--|---------------------------------|-------------------------------|---------------------------------|
|   |                                     | 3.6 $\times$<br>10 <sup>4</sup>                                | 1.2 $\times$<br>10 <sup>4</sup> | 4 $\times$<br>10 <sup>3</sup> | 1.3 $\times$<br>10 <sup>5</sup> |
|   |                                     | <i>cpm <math>\times 10^{-3}</math></i>                         |                                 |                               |                                 |
| Day 0 low density fraction cells, 12% Ia <sup>+</sup>                   | —                                   | 27   | 7                               | 3                             | 3                               |
|   | +                                   | 34   | 12                              | 5                             | 4                               |
| 2-d culture of day 0 low density fraction<br>cells, 28% Ia <sup>+</sup> | —                                   | 204  | 71                              | 22                            | 8                               |
|   | +                                   | 197  | 67                              | 13                            | 7                               |
| As above, plus 5 $\times 10^4$ day 0 low density<br>fraction cells      | —                                   | 118  | 57                              | 25                            | 12                              |
|   | +                                   | 178  | 79                              | 34                            | 14                              |

This experiment shows that fresh low-density cultured epidermal cells, which have weak stimulating activity, do not inhibit active cultured low-density populations, except for a slight indomethacin-sensitive effect at high doses. Two similar experiments have been performed with mixtures of fresh epidermal and splenic DC.

TABLE X  
*Ia<sup>-</sup> Low Density Epidermal Cells Neither Stimulate Nor Amplify T Cell Mitogenesis*

| Stimulator cells                                | [ <sup>3</sup> H]Thymidine with stimulator doses of: |                   |                 |                 |
|---|--|-------------------|-----------------|-----------------|
|   | 3.3 $\times 10^4$                                    | 1.1 $\times 10^4$ | 3 $\times 10^3$ | 1 $\times 10^3$ |
|   | <i>cpm <math>\times 10^{-3}</math></i>               |                   |                 |                 |
| Exp. A  |  |                   |                 |                 |
| 1. Day 2 low density epidermis                  | 236  | 143               | 64              | 25              |
| 2. As above, with Ia <sup>+</sup> cells removed | 2  | —                 | —               | —               |
| 3. Day 0 low density epidermis                  | 13   | 3                 | 2               | —               |
| 4. Group 2 plus group 3                         | 13   | 4                 | 3               | —               |
| Exp. B  |  |                   |                 |                 |
| 1. Day 3 low density epidermis                  | 176  | 92                | 35              | —               |
| 2. As above, with Ia <sup>+</sup> cells removed | 4  | —                 | —               | —               |
| 3. Spleen DC                                    | —  | 119               | 52              | 13              |
| 4. Group 2 plus group 3                         | —  | 111               | 35              | 13              |

Ia<sup>+</sup> LC were removed from cultured epidermis, low density fraction by panning with B21-2  $\alpha$ -Ia, then tested as stimulators of oxidative mitogenesis (compare groups 1 and 2), or as amplifiers of the stimulatory activity of day 0 low density epidermal cells, or spleen DC (compare groups 3 and 4; the latter had a constant dose of 3.3  $\times 10^4$  Ia<sup>-</sup> cells). T cells alone took up <2,000 cpm.

ATPase. As a result, the ultrastructure and phenotype of cultured LC became remarkably similar to lymphoid DC.

Stimulatory capacity for T cell proliferative responses (oxidative mitogenesis and the mixed leukocyte reaction) was monitored daily. Initially, stimulatory capacity was very weak, even though LC expressed substantial levels of Ia antigens. After 2–3 d in culture, LC had become 3–10 times more potent than spleen DC. 30 LC could induce significant responses in cultures of 3  $\times 10^5$  responding T cells. Removal of Ia<sup>+</sup> LC at the start of culture ablated the development of stimulatory activity, but exposure to 1,500 rad of ionizing

irradiation did not. Mixing experiments showed that contaminating Ia<sup>-</sup> epidermal cells did not alter the function of Ia<sup>+</sup> stimulators.

Therefore, LC seem to be immunologically immature, but acquire many of the features of spleen DC during culture. We suggest that functioning lymphoid DC may, in general, be derived from less mature precursors located in nonlymphoid tissues.

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