Further Evidence for the Self-Reproducing Capacity of Langerhans Cells in Human Skin

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The limited number of Langerhans cells (LC) in the epidermis is one of the main reasons for the technical difficulties in resolving the question of LC kinetics. In the present paper, we describe a method to evaluate the LC replication potential in epidermis. The procedure is based on the specific incorporation of bromodeoxyuridine (BrdU), a thymidine analogue, into the DNA during the S-phase of the cell cycle. Mice, bearing human skin grafts, were injected s.c. every 6 h for up to 17 days with BrdU. At different times, the incorporated BrdU as well as the human epidermal LC were revealed on skin sections using anti-BrdU and OKT-6 monoclonal antibodies, respectively. After 6 h, 4.9% of the LC were labeled with BrdU. Then, the number of OKT-6(+) BrdU(+) cells increased in a linear manner and achieved 34% at 120 h, 67% at 240 h, and 94% at 400 h during the course of continuous labeling procedures. Based on this result we calculated a total cell cycle time of 392 h (16.3 days) and 12 h for the S-phase for human epidermal LC.

Applying this technique, we were able to show also that 48 h after local treatment with 12-*O*-tetradecanoylphorbol-13-acetate or after stripping, the number of BrdU-labeled LC was considerably increased. Furthermore, after i.p. injection of colchicine in the nude mouse, human epidermal LC undergoing mitosis were evidenced by electron microscopy in the graft. From these results we conclude that the LC are actively cycling—therewith a self-reproducing cell population in human epidermis. *J Invest Dermatol 88:17–20, 1987*

Our previous flow-cytometric study, performed on normal human epidermal cell suspensions double stained with OKT-6 monoclonal antibody and DNA dye, demonstrated that OKT-6(+) Langerhans cells (LC) may be found in different phases of the cell cycle [1]. Although we showed that the DNA content of human LC varied according to the different cell cycle phases, this study did not answer the question as to whether human epidermal LC may actively cycle in the epidermis.

The detection of replicating cells is usually accomplished by demonstrating [^{3}H]thymidine incorporation into DNA by autoradiography. This method is time-consuming and does not allow investigations of the minor cell populations such as LC, which have to be identified simultaneously by immunologic markers or electron microscopy.

In the present study, bromodeoxyuridine (BrdU), an analogue of thymidine that incorporates specifically into DNA during the S-phase of the cell cycle, was used to estimate the LC replicating potential in human skin grafted onto the nude mouse. An anti-BrdU monoclonal antibody coupled with fluorescein isothiocyanate (FITC) was then applied on skin sections to identify cells having achieved DNA synthesis. A parallel application of OKT-6 monoclonal antibody as a marker of LC allowed us to estimate the number of BrdU-labeled LC. Consequently, the number of LC in S-phase was evaluated at different times during a continuous BrdU labeling procedure (up to 17 days) as well as after stripping or local 12-*O*-tetradecanoylphorbol-13-acetate (TPA) application. In addition, experiments to investigate a LC undergoing mitosis were performed.

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Abbreviations:
BrdU: bromodeoxyuridine
FITC: fluorescein isothiocyanate
LC: Langerhans cell(s)
PBS: phosphate-buffered saline
TEM: transmission electron microscopy
TPA: 12-O-tetradecanoylphorbol-13-acetate

MATERIALS AND METHODS

Continuous BrdU Labeling Procedure Pathogen-free, congenitally athymic ("nude") mice (Swiss, nu/nu, Iffa Credo, Les Onçins, France) were grafted with normal human skin as previously described [2]. Two-month-old grafts were used in the study. Every 6 h during 17 days, 1 mg of BrdU diluted into 0.2 ml of saline was injected s.c. in mice. After various times (6, 12, 24, 48, 72, 96, 120, 180, 240, and 400 h) the animals were sacrificed and the grafts excised. Immediately following removal, they were quickly frozen in liquid nitrogen (0.5 h) and then stored at −80°C until sectioning.

In additional experiments, 48 h before BrdU injection, grafted skin was either treated with 100 nmol of TPA or stripped (20 strips). Six hours after BrdU injection, grafts were removed, frozen, and processed for immunofluorescence study. As control, untreated grafts were used. Two skin grafts originating from different human donors were used to establish LC BrdU incorporation either at each time point of continuous labeling or after local applications.

Immunofluorescence Study Five micron-thick, cryostat skin sections were prepared and an OKT-6 monoclonal antibody (Ortho, Raritan, New Jersey) diluted 1:10 was used for LC staining. This was followed by rabbit antimouse IgG_1 (Nordic, The Neth-

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erlands) diluted 1:30 and tetramethylrhodamine isothiocyanate (TRITC)-conjugated swine antirabbit IgG (Daco, Denmark) diluted 1:100. All incubations lasted 45 min, were followed by 10-min washes in PBS, and performed at room temperature.

To identify simultaneously the cells that incorporated BrdU, the skin sections were treated with 4 N HCl (5 min) followed by 10-min washes in 0.1 M sodium tetraborate, pH 8.5, and thereafter in phosphate-buffered saline (PBS). Then, sections were incubated for 45 min with FITC-conjugated anti-BrdU monoclonal antibody (Becton-Dickinson, Mountain View, California) diluted 1:10. After 2 subsequent washes in PBS, sections were mounted in PBS/glycerol and examined immediately after staining with a Zeiss epi-illumination fluorescence microscope fitted with fluorescein and rhodamine selective filters.

Controls were performed in the absence of either OKT-6 monoclonal antibody treatment or of BrdU injection. As a further control, OKT-6 antibody was replaced by monoclonal antibody to cytokeratin component No. 18 (Boehringer, Mannheim, F.R.G.) which is as OKT-6 monoclonal, a mouse IgG_1 immunoglobulin.

The number of cells that both incorporated BrdU and showed OKT-6 specificity (BrdU + OKT-6+) was related to the total number of OKT-6(+) cells and expressed as a mean percentage. Only OKT-6(+) cells with distinct cellular structure were included and at least 1000 OKT-6(+) cells were counted for each graft.

Transmission Electron Microscopic (TEM) Study and LC Mitotic Activity Investigations To increase the probability of finding a LC in mitosis, TPA-treated human skin grafts were used for TEM examination. In addition, mice were injected i.p. with colchicine (0.1 mg/0.1 ml of saline), a known metaphase blocker [3]. Five hours later the grafts were removed, small strips were fixed, treated with osmium, dehydrated, and embedded in Epon according to routine TEM technique. Thereafter, 0.5–1.0 μm-thick sections were prepared, stained with 1.0% toluidine blue, and observed by light microscopy (× 630). All suprabasal cells that showed a clear cytoplasm and mitotic figures were noted. Subsequently 300–500 Å-thick sections were prepared to observe these cells under the electron microscope (Jeol 1200 Ex.).

RESULTS AND DISCUSSION

In previous studies it has been documented by Krueger et al [4,5] as well as by the authors [2] that the LC in the human skin grafted onto nude mouse represent a stable cell population during a long period of time (we accomplished our studies after 9 months). In addition, these LC continued to express their specific membrane antigens (HLA-DR and T-6) and penetration of murine LC into the human grafts was not observed. We used this model for biologic investigations of LC to elucidate cell cycle properties of LC.

In human epidermis, which is multilayered, LC are found at two levels. The majority of them are observed in the higher part of the epidermis, just below the stratum granulosum. Other LC are situated in the lower third of the epidermis, in basal or just suprabasal position. In human skin grafts on nude mouse, the same LC distribution was observed. The continuous labeling experiments demonstrated that at the beginning (up to 24 h) the double-stained (BrdU + OKT-6+) LC were more frequently found in the lower part of the epidermis. Thereafter, equal numbers of double-labeled LC were observed in the lower and higher regions of epidermis (Fig 1). In addition, double-labeled LC were often seen in pairs as has been already reported [6].

As demonstrated in Fig 2, the number of LC that incorporated

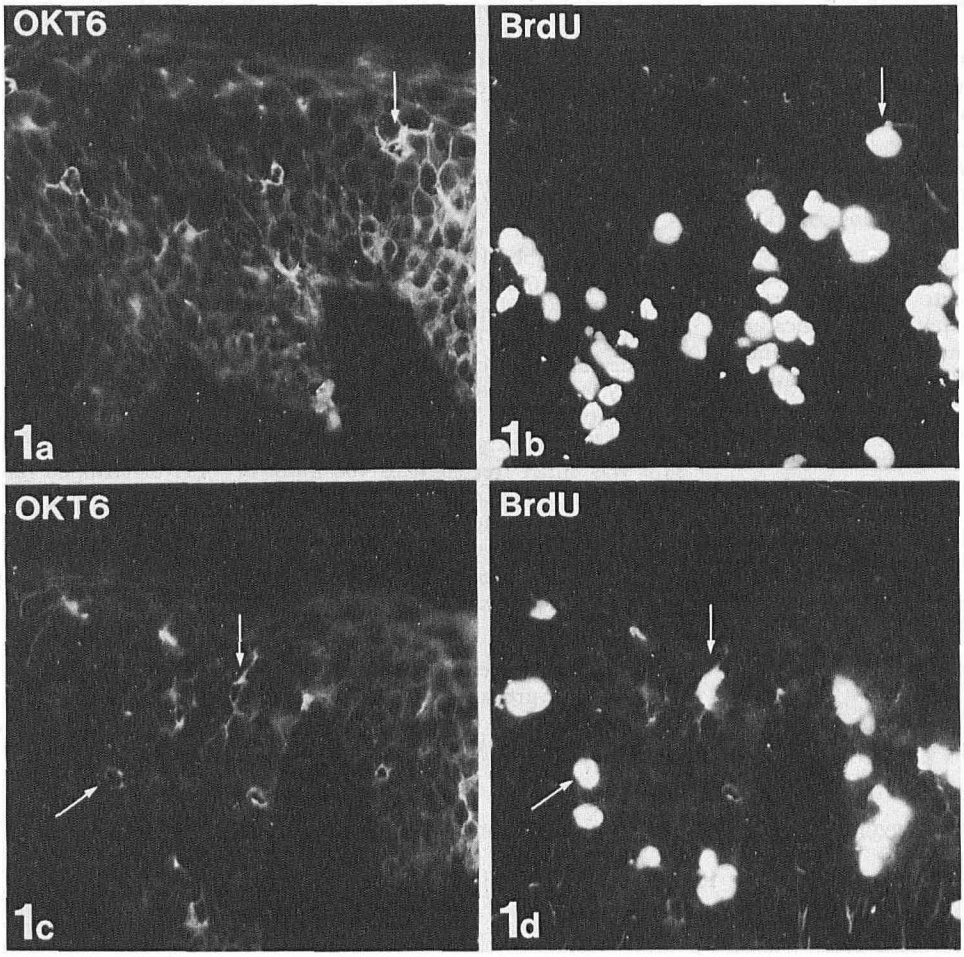

Figure 1. Double-stained skin sections after 96 (a,b) or 72 (c,d) h of repeated BrdU injections. *a and c,* OKT-6 monoclonal antibody was followed by rabbit antimouse IgG_1 and TRITC-conjugated swine antirabbit IgG. *b and d,* After HCl treatment and pH equilibration, incorporated BrdU was revealed with FITC-conjugated anti-BrdU monoclonal antibody. *Arrows* indicate double-stained LC.

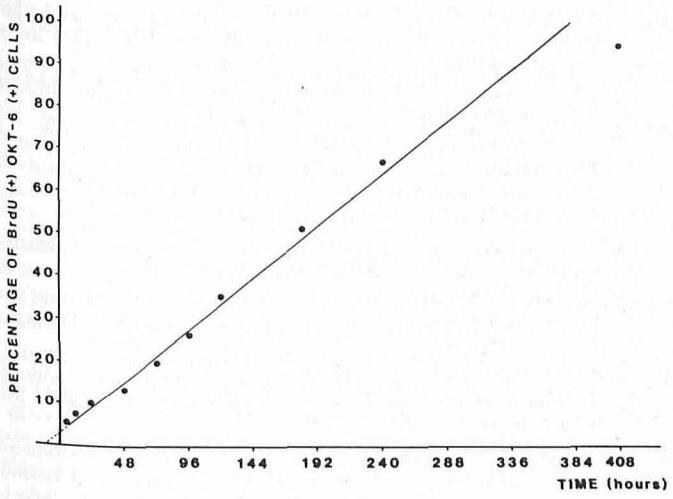

Figure 2. Percentage of BrdU-labeled LC after repeated BrdU injections. BrdU was injected s.c. every 6 h into the mouse bearing human skin grafts. Its incorporation into human LC (OKT-6+ cells) was measured on skin sections at 6, 12, 24, 48, 72, 96, 120, 180, 240, and 400 h. Each *point* represents the mean of 2 measurements on different human skin grafts.

BrdU increased in a linear manner during the course of continuous labeling experiments. The characteristics of the curve obtained with the 17-day experiment suggest that all LC are cycling. Assuming a homogenicity of LC population, different parameters of the LC cell cycle can be read from the graph and calculated, therefore, with the procedure described by Potten and Wichmann [7]. Accordingly, the total cell cycle time for LC is 392 h (16.3 days) and the duration of the S-phase is 12 h. Finally, 3.1% of LC would be in S-phase at time zero, in agreement with our recent findings showing the mean percentage of LC in S-phase to be 2.9 ± 0.8% as evaluated by flow cytometry of fresh human epidermal cell suspensions [1].

In previous studies, [^{3}H]thymidine incorporation into the nuclei of epidermal LC was observed but labeling indices were low [8,9]. These studies utilizing combined autoradiography and cytochemistry suffer, however, from the uncertainty of LC identification. The method that we applied in the present study has been used to date for flow cytometric measurement of cell DNA replication [10,11]. We adopted this method for our purpose using in vivo BrdU injections. Cells that incorporated BrdU were revealed on skin sections by anti-BrdU monoclonal antibody coupled with FITC. To limit our evaluations to the LC, OKT-6 antibody, a highly specific marker of human LC [12] was used. This immunologic method proved to be adequate since, in control sections, no cross-reactivity between the antibodies could be demonstrated. Indeed, when OKT-6 antibody was suppressed in the series of reagents or replaced by a monoclonal antibody of the same immunoglobulin class (IgG_1) which does not react with adult human skin, no labeling of LC was observed. Moreover, in skin sections of animals not injected with BrdU, the nuclear staining was absent. Furthermore, the use of HC1 to denaturate the cellular DNA, so that the monoclonal anti-BrdU can bind to the incorporated BrdU (the antibody does not bind to BrdU in double-stranded DNA), did not substantially alter the TRITC-OKT-6 fluorescence distribution.

The nuclei of OKT-6(−) cells (keratinocytes) were also heavily stained with anti-BrdU antibody and their number increased during progression of the continuous-labeling procedure. We estimated the number of BrdU-labeled basal cells only at days 2 and 4. The counts obtained (13% and 18%, respectively) were comparable with those reported by Briggaman and Kelly [13] using the same human skin model, but continuous [^{3}H]thymidine incorporation.

Table I. Mean Percentage of BrdU-Labeled Langerhans Cells in Control, Stripped, or TPA-Treated Human Skin Grafts

Control	Stripping[a]	TPA Application[b]
4.9	8.2	11.2

[a]Transplanted human skin was stripped 20 times with a transparent adhesive tape and BrdU was injected 48 h later. Grafts were excised 6 h after BrdU injection, and the percentage of OKT-6 + BrdU + cells was evaluated.

[b]One hundred nanomoles of TPA in 15 μl of acetone was applied topically and BrdU was injected 48 h later. Grafts were excised 6 h after BrdU injection, and the percentage of BrdU-labeled LC was evaluated.

It was interesting to investigate the influence of chemical or mechanical stimuli on LC kinetics. For this purpose, either TPA or stripping were applied and the number of LC in S-phase (OKT-6 + BrdU + cells) was estimated after 48 h. The period of 48 h was chosen because maximal increase of epidermal proliferation following stripping or TPA application on human skin grafted onto the nude mouse was observed at this time [14]. As demonstrated in Table I, TPA treatment induced a more than 100% increase of LC in S-phase. Stripping was less effective; nevertheless, an increase from 4.9% to 8.2% of BrdU(+) LC was also observed here, confirming the data reported by Gschnait and Brenner [15] o. guinea pig LC.

Interestingly, whatever the cell type, the proliferation control seems to be exhorted at the level of the tissue.

The mitotic activity of LC in the epidermis has been reported in only a few studies [16,17]. These rather accidental observations of LC in mitosis are probably due to the rarity of these cells in the epidermis as well as to the short time in which mitosis is accomplished. Thus, the probability of finding a LC in mitosis is extremely low. In the present study, to increase this probability, we used TPA-treated grafts as well as injection of colchicine to block cells in metaphase. Then, semithin skin sections were observed by light microscopy and selected areas processed for electron microscopic examination. Among 1000 LC (in suprabasal position, clear cytoplasm) that we observed (magnification × 630), 4 cells presented mitotic figures. All 4 of these cells under

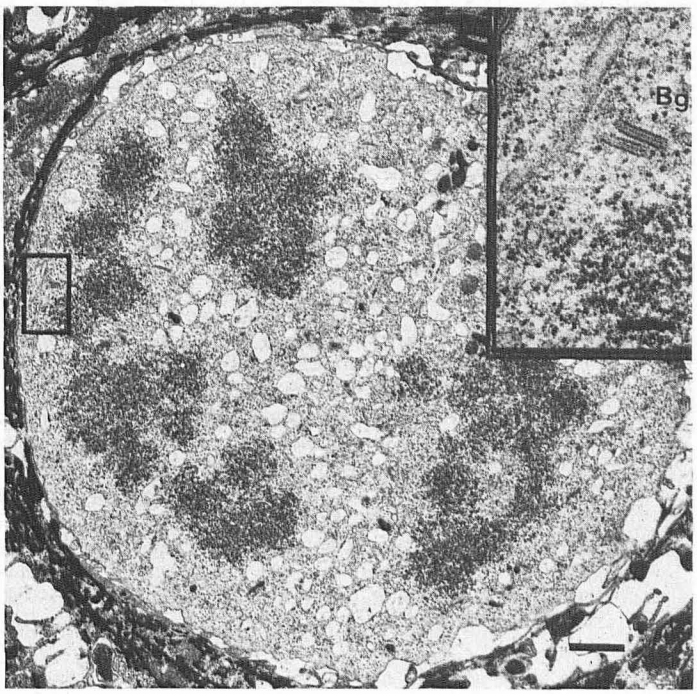

Figure 3. Electron micrograph of LC undergoing mitosis. *Bar* = 1 μm. *Inset, Bg* = Birbeck granules. *Bar* = 0.2 μm.

the electron microscope were found to contain Birbeck granules and, therefore, to be LC. One of these cells is shown in Fig 3. It presents dense clumps of chromatin; the nuclear envelope has already disappeared and the nucleolus is no longer distinct. This state corresponds to either late prometaphase or early metaphase.

It has been documented in mice [18,19] as well as in humans [20,21] that epidermal LC originate from the bone marrow. Katz et al [19] suggested that LC are not a stable resident cell population of the epidermis but migrate from the bone marrow into the skin, where they are continuously replaced at an as yet unknown rate. Accordingly, the observations of LC crossing the dermal-epidermal junction as well as their presence in the dermis has been explained by their migration into the epidermis [22]. Nevertheless, the inverse direction from the epidermis to the dermis has also to be considered [23]. Bearing in mind also the antigen-presenting capacities of LC and their immunologic role in the epidermis [24], the movement of LC from the epidermis to the dermis and adjacent lymph nodes seems to be more probable. Moreover, the large body of evidence already cited as well as the present study demonstrate that LC are an actively cycling (passing through the S-phase and cell division) cell population in normal epidermis. Furthermore, we also demonstrate in this study that all LC are cycling and that they are a stable, self-reproducing cell population in normal epidermis.

In addition, we report for the first time an estimation of the LC cycle parameters. The total cell cycle time of LC we extrapolated from our data (16.3 days) is nearly twice that reported for human keratinocytes [25,26], indicating that LC are a rather slow-cycling cell population. Accordingly, the observations that a relatively long period is necessary for the PUVA (psoralen plus ultraviolet A)-treated skin to regain the normal population of LC [27,28] are in agreement with this later finding.

It should be mentioned finally that the above observations are not incompatible with the fact that LC migrate, in addition, from the bone marrow, e.g., under circumstances other than embryogenesis such as bone marrow reconstitution of lethally irradiated subjects. It may be suggested also that probably other toxic stimuli such as serious thermal injury and consequent loss of the epidermis may induce LC production in the bone marrow and subsequently invoke their migration into the skin. However, once LC reach the skin they constitute a stable, self-reproducing cell population, which seems to be submitted to the same local proliferation controls as the major epidermal cell component keratinocytes.

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