DISTRIBUTION AND TURNOVER OF LANGERHANS CELLS DURING DELAYED IMMUNE RESPONSES IN HUMAN SKIN

By GILLA KAPLAN,* ASMA NUSRAT,* MARGIT D. WITMER,* INDIRA NATH,$ AND ZANVIL A. COHN*

From *The Laboratory of Cellular Physiology and Immunology of The Rockefeller University, New York, 10021; and $The All-India Institute of Medical Sciences, New Delhi, India

The generation of a delayed immune reaction in the skin involves a large number of complex events leading to the emigration and activation of blood-borne and local elements (1–3). This laboratory has over the past two years been involved in the analysis of such reactions in the context of leprosy. The lepromatous state is characterized by a lack of specific responsiveness to Mycobacterium leprae antigens, the absence of the T helper subset from dermal lesions, and the unrestricted proliferation of M. leprae in dermal macrophages (4–7). To analyze the striking emigratory defects in these patients, we have generated a second-party immune response in the skin with purified protein derivative of tuberculin (PPD). In many sensitized individuals this has lead to a massive influx of monocytes and T cells, largely of the helper phenotype, with subsequent changes in epidermal thickness and la expression (8).

In the course of these immunocytochemical studies, we noted alterations in the number and distribution of T6+ Langerhans cells (LC)1 in both the epidermis and dermis. These cells play important accessory roles in initiating T cell replication and lymphokine formation (9–11). Their distribution and turnover in the skin is poorly understood. For this reason we have carried out a combined light and electron microscope study, sampling PPD reactions in leprosy patients at short intervals from 1–14 d after the administration of antigen. This has allowed us to quantify the number of LC, evaluate their directional flux into and out of the dermis and epidermis, determine nearest neighbors, and make predictions as to their fate.

Materials and Methods

Generation of Delayed-type Hypersensitivity Response to PPD. After informed consent was obtained, we evaluated the delayed response to 5 U of PPD in 65 Indian lepromatous leprosy patients from New Delhi (a high endemic area for tuberculosis). The study group included 45 lepromatous (LL) (see diagnosis below) and 20 borderline lepromatous leprosy patients (BL) (see below). In addition, 57 tuberculoid patients and 50 nonleprosy controls were tested for their responses to intradermal PPD but were not biopsied. The leprosy patients were examined in collaboration with Drs. A. K. Sharma and R. S. Mishra, at the Department of Dermatology, Safdarjung Hospital, New Delhi, India. Antigen was injected intradermally into the skin of the back. Two 4-mm punch biopsies, one from the PPD-
injected site and one from an uninjected adjacent site, were taken at 18, 42, 68-72, and 96 h, and at 1 and 2 wk after injection from consenting LL and BL patients. The biopsies were fixed as described below and transported to the U. S. for further processing.

**Leprosy and Leishmaniasis Patients.** After informed consent was obtained, skin biopsies from 16 untreated patients from Brazil and 6 untreated patients from Colombia with various forms of leprosy were collected and examined. The Brazilian patients were examined in collaboration with Dr. E. N. Sarno of the Department of General Pathology, Hospital de Clinicas, Universidade do Rio de Janeiro, Brazil. The Colombian patients were studied in collaboration with Dr. U. Elvers of the Federico Lleras Acosta Hospital of Dermatology, Bogota, Colombia. Clinical diagnosis was accompanied by a histopathological diagnosis established by Dr. I. Nath, Dr. E. N. Sarno, and Dr. C. K. Job at the U. S. Public Health Service Gillis W. Long, Hansen’s Disease Center, Carville, LA, according to the Ridley Jopling classification (12). Patients with cutaneous leishmaniasis were studied in collaboration with Dr. W. M. Rojas and Drs. M. I. Restrepo and F. M. Restrepo, at the Corporacion para Investigaciones Biologicas and The Regional Health Laboratory, Medellin, Colombia. After informed consent was obtained, 4-mm punch biopsies were taken from the periphery of the lesions of five patients.

**Fixation and Processing of Cutaneous Biopsies.** Skin biopsies were fixed in paraformaldehyde (3%), lysine (0.075 M) and sodium m-periodate (0.01 M) in PBS for 4 h at 4°C as described by McLean and Nakane (13). This fixative preserves structural details without inhibiting the binding of monoclonal antibodies to their respective antigens (14). The biopsies were stabilized for freezing by overnight washing in PBS containing sucrose (10%) and digitonin, and then serially suspending them in graded solutions of sucrose (15-25%). The biopsies were stored in PBS with sucrose/glycerol (25 and 5%, respectively) until frozen.

**Immunocytochemical Staining of Sections.** The biopsies were embedded in OCT compound and frozen at −20°C. 6-8-μm sections were cut on a cryostat and applied to gelatin-coated multwell slides (Carlson Scientific, Inc., Peotone, IL). The sections were dried overnight at 37°C, rehydrated in PBS, and incubated with mouse monoclonal antibodies followed by biotinylated horse anti-mouse Ig, and then avidin-biotin peroxidase complexes (Vector Laboratories, Burlington, CA). The reaction product was developed with 0.4 mg/ml diaminobenzidine in 0.02 M Tris buffer, pH 7.6, or 0.8 mg/ml 3-amino-9-ethylcarbazole and 0.03% or 0.015 M H2O2, respectively. Sections were counterstained with hematoxylin.

**Monoclonal Antibodies.** Mouse anti-human monoclonal antibodies were used for the identification of specific cell types. OKT6 (anti-thymocyte and Langerhans cells) was obtained from Ortho Diagnostics Systems Inc. (Raritan, NJ) (15). 9.3F10 (anti-HLA class II) was produced in this laboratory (16). The cell line 63D3 (anti-monocyte/macrophage) was obtained from The American Type Culture Collection, Rockville, MD (17). Leu-1 (anti-pan T lymphocyte), Leu-2a (anti-suppressor/cytotoxic T lymphocyte), and Leu-3a (anti-helper T lymphocyte) were obtained from the Becton Dickinson Monoclonal Center (Mountain View, CA) (18-19).

**Evaluation of Staining and Morphological Changes.** Adjacent sections were evaluated for specific cell staining with a Nikon Microphot-FX light microscope. The numbers of T6+ cells of the epidermis and the dermis were evaluated by direct counting of the stained cell bodies (with nuclei) per 40X microscopic field. The total area of epidermis and upper dermis were examined on each section, T6+ cells counted, and the mean and standard deviation of the mean calculated. At least four different sections, with about 10 adjacent 40X microscopic fields from every biopsy were counted. To distinguish between the brown stain of diaminobenzidine and melanin, additional sections from all the biopsies were stained with aminoethylcarbazole (which gives a red stain) and counted. No significant differences between the numbers of T6+ cells in the epidermis of sections stained with the two substrates were observed. However, sections stained with aminoethylcarbazole were easier to count and did not require the use of phase-contrast microscopy, as did the counting of sections stained with diaminobenzidine. Photography was carried out on the sections stained with aminoethylcarbazole.

**Electron Microscopy.** A part of each biopsy and sections stained with OKT6 as above
were processed for transmission electron microscopy studies. Biopsies were washed in saline at 4°C, cut into 1–2-mm pieces and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 0.1 M sucrose, pH 7.4, for 16 h at 4°C. Stained frozen sections were postfixed as above (20). The tissues were cut to 1-mm or smaller and postfixed in 2% OsO₄ for 6 h at 4°C. The tissue was then stained en bloc for 2 h with 0.25% uranyl acetate, dehydrated in increments with ethanol, and embedded in epon blocks. Semithin sections were stained with methylene blue–azur–basic fuchsin and examined for areas containing infiltrating cells. Sections were stained with uranyl acetate and lead citrate, and examined with a Jeol JEM 100CX transmission electron microscope. At least 200 cells from each patient were examined and photographed on Kodak electron image film.

Result

Temporal Distribution of Epidermal LC. The delayed immune response to PPD was examined by daily sampling of sites and immunocytochemical analysis of the distribution of Ia and T6 antigens. As reported previously, keratinocytes increased in number and size as the epidermis over the infiltrated PPD injection site thickened during the first 72 h (8). Accompanying this proliferative event was the progressive expression of Ia antigen on the surface of keratinocytes. These changes were first observed at ~24 h as the rete elongated and their cells enlarged (not shown).

Before the administration of antigen, LC were the only Ia⁺ elements in the epidermis. These cells, which also stained selectively for the T6 antigen, were evenly distributed within the thin epidermis, exhibiting long dendritic projections from a central cell body (Fig. 1a). With the proliferation and Ia expression on keratinocytes, this antigen was no longer a distinctive feature of LC. Identification of this cell type therefore depended on the T6 marker during the remainder of the immune response. As keratinocyte proliferation and epidermal thickening occurred, a dramatic change took place in the distribution of LC, as illustrated in Fig. 1. T6⁺ LC were displaced to the upper zone of the thickened epidermis, leaving a basal portion relatively devoid of this cell population. By 42 h T6⁺ LC were tightly packed just beneath the stratum corneum and by 72 h LC were virtually absent from the thickened epidermis. After 7 d T6⁺ cells started to reappear in the epidermis and by 14 d normal numbers of T6 cells were evenly distributed in the epidermis in many patients. By this time the thickened epidermis had differentiated, sloughing a cornified layer and once again taking on a pre-antigen stimulation contour. Biopsies taken from patients with negative PPD responses (<10 mm) failed to show either the epidermal keratinocyte changes or the redistribution and loss of LC.

Temporal Distribution of Dermal LC. Before administration of PPD, the dermis of lepromatous leprosy patients had an occasional T6⁺ cell (1.8 ± 1.6 T6⁺ cells per 40X field) associated with the loose macrophage infiltrate. After antigen administration and the initiation of the immune response, larger numbers of T6⁺ cells appeared in association with the new wave of mononuclear leukocyte emigration (Fig. 2). Their numbers peaked at 72 h, where they were in close association with the preponderant helper T cell subset localized only to the superficial dermal infiltrates, as shown in Fig. 3. By 7 d the T6⁺ cells of the dermis had fallen to control values. At 2 wk, a time in which the epidermis was being repopulated, many T6⁺ cells were seen immediately below the basal layer of the epidermis (Fig. 1f).

Identification of the Dermal T6⁺ Cells by Immunelectron Microscopy. Iden-
766 LANGERHANS CELLS DURING DELAYED IMMUNE RESPONSES

**Figure 1.** Effect of intradermal tuberculin on the distribution of T6+ LC in the epidermis of LL patients. Photomicrographs of anti-T6 (OKT6) monoclonal antibody staining of the epidermal cells. (a) Uninjected lesion: T6+ LC are evenly distributed throughout the epidermis. (b) At 24 h after intradermal PPD, a slight elongation of the rete is observed, but distribution of the T6+ LC has not changed. (c) At 41 h the keratinocytes of the rete are enlarged, the rete are more elongated than in controls, and the T6+ LC have redistributed to the upper epidermis. (d) At 91 h the epidermis is clearly thickened and no T6+ LC are found. (e) At 7 d the upper epidermis has cornified and is sloughing off. T6+ LC are not seen. (f) At 14 d T6+ LC have repopulated the epidermis and the epidermis has returned to normal thickness. Some T6+ LC are seen just below the epidermis (arrows). The dark areas in the basal layer of the epidermis are due to melanin granules.

tification of the dermal T6+ cells that entered the antigen-induced infiltrate as typical LC was established by a strict correlation between the presence of Birbeck granules and plasma membrane T6 antigen by electron microscopy. These cells were always perivascular and closely associated with T cells at the 72–96-h time
interval (Figs. 4 and 5). At this time the overlying epidermis was virtually free of T6+ cells containing Birbeck granules, in keeping with the immunocytochemical analysis at the light microscope level (Fig. 1). There were no cells in either the dermis or epidermis that contained Birbeck granules and lacked the T6 antigen.

Quantitative Analysis of the T6+ Populations in the Dermis and Epidermis. The qualitative changes in the number and distribution of LC during the course of a delayed immune response in the skin required additional analysis. For this purpose, biopsies from five to nine patients, obtained at time intervals ranging from 18 h to 2 wk after administration of PPD were analyzed. Multiple sections from each biopsy were stained for the T6 antigen with the immunoperoxidase technique, and the total number of LC was determined. The results of this analysis are seen in Fig. 6. The mean number ± one standard deviation of T6+ cells in the un.injected skin of patients taken during the course of the reaction is shown by the line and shaded area.

The epidermis of the un injected skin contained 9.6 ± 1.4 LC per 40X field, and these were evenly distributed throughout its depth (Fig. 6a). During the first 48 h of the reaction only small decrements were noted in the total cell numbers, even though striking alterations were occurring with the keratinocytes and the distribution of LC. In particular, as keratinocyte proliferation ensued and the epidermis increased in thickness, LC were segregated in the uppermost
superficial zone. Suddenly, at 72 h, the total T6+ cell count fell precipitously to values <20% of controls. Thereafter, T6+ cell levels remained low in the epidermis even though keratinocyte proliferation continued for some days. Slowly, the epidermis was repopulated with new T6+ LC, and by 2 wk their numbers were approaching normal values.

The dermal reaction exhibited quite a different pattern. Initially only a few T6+ LC were found in the basal infiltrate of lepromatous skin (Fig. 6b). After the administration of antigen, many T cells and monocytes entered the site during the first 48 h, reaching maximum values by 3–4 d. Associated with this influx was the presence of increased numbers of T6+ LC, which reached peak numbers during the same 3–4-d period. These cells were clustered in the T-rich areas of the infiltrate, most of the cells being of the T4 helper phenotype. Relatively few dermal LC were seen in the clear zone lying just beneath the epidermal retes during this phase of the response. In fact, this zone was relatively devoid of all cells during the early phases of the immune response. By 1 wk after antigen administration the number of dermal LC had regressed to control levels even though significant numbers of T cells and monocyte/macrophages still populated the site of the immune response. At 2 wk, dermal LC were again elevated, however, their distribution was quite different from the early wave of cells. They were now more evenly distributed in the upper clear zone of the dermis, at a time when repopulation of the epidermis was taking place (Fig. 1f).
Figure 4. Perivascular distribution of T6+ LC. (a) Photomicrograph of T6 staining in a 72-h PPD-induced perivascular infiltrate in the upper dermis. The central vessel of the infiltrate is seen (arrow). X300. (b) Electron microscopic photomicrograph of T6-stained cells in a 72-h PPD-induced infiltrate. V, small vessel; T, T lymphocytes; LC, Langerhans cell with T6 staining (arrows). X5,200.

Presence of T6+ Cells in Lesions of Untreated Leprosy and Leishmaniasis Patients. The epidermal and dermal modifications associated with the generation of delayed reactions in the dermis of LL patients suggested that similar changes might occur in the lesions of tuberculoid leprosy. Biopsies were taken from the lesions of 10 untreated polar and borderline (BL) forms of the disease. Tuberculoid and borderline tuberculoid patients exhibited T6+ cells in the granulomas of the upper dermis (five to six cells per 40X field; not shown). None of the biopsies from polar and borderline lepromatous patients showed more than one or two T6+ cells per 40X field in the dermis. Thickening of the epidermis overlaying the lesions were observed in many of the tuberculoid leprosy lesions, but no reduction in the numbers nor changes in the distribution of the T6+ cells was observed in the epidermis overlying these lesions (not shown).
FIGURE 5. Immunoelectron microscopic photomicrographs of T6 staining of LC of the dermis in a 72-hour PPD-induced response. (a) T6 staining (arrows) of LC in the epidermis of an uninjected lesion. The section was not stained with uranyl acetate and lead citrate to facilitate easy identification of the membrane T6 stain. ×7500. (b–e) Perivascular (V) Langerhans cells (LC) in close contact with T cells (T). T6 stain (arrows) and Birbeck granules (*) are seen in the LC. (b) ×8300. (c) ×1000. (d) ×16500. (e) ×40000.
A large accumulation of mononuclear cells also occurs at the edge of the lesions of cutaneous Leishmaniasis (Fig. 7). Many of the cells in these lesions are T lymphocytes of both T4 and T8 phenotype, often with a majority of T8+ cells (Fig. 7, e and f). In many of the untreated lesions the keratinocytes are Ia+ (Fig. 7a). Examination of biopsy specimens from five Colombian patients revealed extensive epidermal thickening, with large numbers of T6+ LC in the epidermis over the area of dermal infiltrate (Fig. 7, b and c). Each of the five patients showed strongly staining T6+ LC in the granulomas of the upper dermis (Fig. 7d). As in the PPD responses and the lesions of tuberculoid leprosy, the LC appeared perivascular and in association with T cells. In both tuberculoid leprosy and cutaneous Leishmaniasis, the exuberant T cell and monocyte response in the dermis and subsequent alteration of the epidermis were associated with the presence of larger numbers of dermal LC.

Discussion

A number of factors have influenced our ability to arrive at a qualitative and quantitative analysis of the distribution and fate of LC during a delayed immune response. The first of these is the synchrony of the immune response to intradermal antigen and its persistence for a finite time. Within 24 h, helper T cells and monocytes have accumulated perivascularly, and presumably are secreting a variety of lymphokines and monokines. From the expression of class II MHC antigens on the overlying keratinocytes (8) we conclude that IFN-γ is included in this repertoire. The second factor is the use of the rather stable T6 marker at both the light and electron microscopic levels, and its correlation with Birbeck granules (21). Prior investigations have often used surface ATPase activity, which
The distribution of T6+ LC in cutaneous Leishmaniasis lesions. (a) Photomicrograph of cells stained with anti-la (9.3) monoclonal antibody. The epidermal keratinocytes and some of the infiltrating cells of the dermis are clearly stained. (b–d) Cells stained with anti-T6. T6+ LC are seen evenly distributed in the epidermis (b and d) and in the upper infiltrates of the dermis (b and c). (e) Cells stained for the helper subset of T cells (Leu-3a). (f) Cell stained for the suppressor/cytotoxic subset of T cells (Leu-2a). More Leu-2a+ cells are observed than Leu-3a+ cells. (a–b) ×75. (c–f) ×200.

is an unstable and nonspecific marker that also stains macrophages (22–24). With this information, gathered at short intervals throughout the PPD response, we have constructed a scenario of the life history of LC, which is presented in Fig. 8.

The emigration of T cells and mononuclear phagocytes leads to the production of epidermal growth factors, which in time result in keratinocyte proliferation and epidermal thickening (8). LC normally distributed throughout the epidermis appear to be entrapped among the enlarging keratinocytes, and as cornification proceeds, they are elevated into the uppermost portions of the skin. This is
The life history of cutaneous LC during a delayed immune response. Epidermis (left to right): Irregularly shaped T6+ LC (bold outline) are initially distributed evenly among keratinocytes. Concomitant with keratinocyte proliferation and epidermal thickening, LC are elevated in the centrifugally moving epidermis. At ~72 h, as differentiation proceeds, these cells are sloughed into the environment. Within another 10 d the epidermis regresses to normal thickness and is repopulated with cells from the upper dermis. Dermis (left to right): Within 24 h after initiating the immune response, large numbers of T helper cells and monocytes accumulate perivascularly. Here, LC leave the microvasculature and appear in the focal infiltrate. Later, as the immune response wanes, all cells of the immune response, including LC, are reduced in number. We postulate their removal via afferent lymphatics and disposition in the local nodes. Later in the response, LC are present high in the dermis and epidermis, and repopulate the epidermis within 2 wk.

maximal at 48–72 h, a time when no significant change in the total number of epidermal LC has occurred but in which the basal zone of the epidermis is relatively free of LC. Abruptly, at 72 h the total cell counts fall and the thickened epidermis becomes almost devoid of T6+ LC. The most likely explanation of these results is the sloughing of LC along with the differentiated keratinocytes, rather than migration of cells into the dermis. All changes occur only in that segment of the epidermis overlying the dermal immune response.

Initially, the dermis of lepromatous patients has only a sparse infiltrate and only rare T6+ cells. As the antigen-induced emigratory response takes place, mononuclear cells form spherical infiltrates in what appears to be a perivascular distribution. Within these foci, large irregular T6+ cells appear in close proximity to the T cells, which are mainly of the T4 phenotype. These cells have the typical features of LC at the electron microscopic level. One possibility is that they arrive from a blood-borne compartment, presumably of bone marrow origin (25), and differentiate at the local site. From the studies of others (11), LC are very potent accessory cells capable of stimulating T cells to replicate and form secretory products. In this particular milieu they are in an excellent position to stimulate T cells and lead to lymphokine production. Shortly after reaching a maximum value at 72 h, their numbers decrease and are then enriched by a second wave of emigratory cells. These new LC are not retained in the local infiltrate but are seen in the dermis adjacent to the epidermis and in the epidermis itself. This localization suggests that they serve to repopulate the epidermis, which by then has the contour of unstimulated skin. As the number of dermal inflammatory cells wanes so does the number of T6+ LC. We suspect that a large segment are transported via afferent lymphatics to the local nodes, and this is in keeping with
the observations on the veiled-dendritic cells of this lymphatic compartment (26, 27). In tuberculoid leprosy and cutaneous Leishmaniasis, where the dermal inflammatory response is chronic and the infiltrates are not cleared, T6+ LC remain in association with the T cells of the lesions (32). T6+ cells are only observed in the infiltrates of the upper dermis in these lesions as well; thus it is possible that at least some of the dermal T6+ LC are on their way into the epidermis.

With this reconstruction in mind we must postulate a number of factors that control the compartmentalization and movement of the intravascular precursor cell. First, some stimulus generated in the immune response must serve to initiate their extravascular migration. Initially, many of the newly emigrated LC remain within inflammatory foci and may well form firm clusters with T cells as they do in vitro (28, 29). Somewhat later, as the intensity of the dermal response declines, we begin to see cells entering and taking up residence in the epidermis. We suspect that this directed movement into the epidermis is controlled by specific chemotactic factors, the most likely origin of which are the keratinocytes themselves. The appearance of T6+ cells only in the infiltrates of the upper dermis strengthens this theory.

Although this information has been gained from the study of a delayed-type immune response, we see no serious objections to this scenario for the steady-state turnover of LC. This postulate suggests that the turnover of LC is linked to that of keratinocytes. Calculations of Bos and Kapsenberg (30) indicate that the total number of epidermal LC in human skin is approximately $0.9 \times 10^9 = (2 \times 10^6 \text{ mm}^2)(450 \text{ LC/mm}^2)$. From the reappearance of epidermal LC after PUVA therapy, Freidman et al. (23) have calculated that normal numbers repopulate the epidermis with an average period of 2 wk (1–3 wk after completing therapy) or an average input of about 45 LC/mm$^2$/d. To repopulate the entire epidermis would take $\sim 20$ d. These rough values are within the range of the time necessary for a basal keratinocyte to reach the uppermost layer, $\sim 14–20$ d (reviewed in 31). Such calculations assume a minimal contribution from local cell division and no large loss of epidermal cells into the dermis. The above rates of repopulation of epidermis (45 LC/mm$^2$/d) would require $45 \times 10^6$ LC/d to maintain steady-state values. Assuming the presence of at least this number of precursors in the circulation, there should be $\sim 6,500$ cells/ml of blood or 0.05% of the total circulating white blood cells.

Summary

The changes in distribution and turnover of T6+ Langerhans cells (LC) in the skin during delayed immune responses to tuberculin, and in the lesions of tuberculoid leprosy and cutaneous Leishmaniasis were investigated. In each situation, there was a dermal accumulation of monocytes and T cells and epidermal thickening with keratinocyte Ia expression. In the tuberculin response a dramatic change in the distribution of LC was observed. By 41 h, T6+ LC were displaced to the upper zone of the thickening epidermis followed by an almost complete loss of LC from the epidermis by $\sim 72$ h. After 7 d, T6+ cells started to reappear in the epidermis, which regained almost normal numbers of T6+ LC by 14 d. After antigen administration and initiation of the delayed immune response, enhanced numbers of T6+ cells appeared in association with the
mononuclear cell infiltrate of the upper dermal lesions. Their numbers peaked by 72 h, were reduced at 7 d, and again enhanced by 14 d, when the epidermis was being repopulated. Similar numbers of T6⁺ cells were found in the chronic lesions of tuberculoid leprosy and cutaneous Leishmaniasis but not lepromatous leprosy. The cells of the dermis were identified as typical LC by the presence of Birbeck granules and surface T6 antigen at the electron microscope level. These cells were closely associated with lymphocytes. We have quantified the number of LC, evaluated their directional flux into the epidermis and dermis, determined nearest neighbors, and made predictions as to their fate.

We thank Dr. R. M. Steinman for his helpful discussion of the project and manuscript. Thanks are due to Judy Adams for her help with the photomicrographs and Linda Rubano for help in typing the manuscript.

Received for publication 13 November 1986.

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