

OSMIUM ZINC IODIDE REACTIVE SITES IN THE EPIDERMAL LANGERHANS CELL

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ABSTRACT

Fixation of epidermis with a mixture of osmium tetroxide and zinc iodide ($\text{OsO}_4\text{-ZnI}_2$) for 24 hr renders the central periodic lamella of the Langerhans cell granule (LCG), the Golgi region, and the nuclear envelope of epidermal Langerhans cells preferentially visible. The use of this technique on Langerhans cells in normal epidermis and in epidermis of patients with histiocytosis (Letterer-Siwe disease) allows a broader visualization of the LCG's than was heretofore possible with routine glutaraldehyde-osmium tetroxide fixation and uranyl acetate-lead staining. The identical staining of Golgi apparatus and LCG favors the view that there is close relation between the Golgi area and the LCG's. Different staining characteristics of the LCG's near the Golgi region and at the cell periphery, respectively, may suggest that the LCG undergoes changes on its way from the Golgi area towards the extracellular space. The hypothesis is advanced that the material which is heavily impregnated with metal after fixation with $\text{OsO}_4\text{-ZnI}_2$ might be a lipid.

INTRODUCTION

The Langerhans cell was first described in human epidermis by Langerhans in 1868 (11) as a "star-shaped" dendritic cell in a suprabasal position which stained deeply after gold impregnation. Since the original description, a variety of studies have been conducted at both the light and electron microscopic levels to determine the origin and function of the Langerhans cell (1, 3, 4, 10, 15-17, 21-27). Of particular interest has been an intracellular organelle, the Langerhans cell granule (LCG), which has been described as characteristically rod-shaped or racquet-shaped (25).

By employing $\text{OsO}_4\text{-ZnI}_2$, a stain used in light microscopy for epidermal dendritic cells (6), it is possible to observe at the ultrastructural level a heavy and preferential deposition of metal on the

LCG's and the Golgi region in Langerhans cells of normal human epidermis and of epidermis of a histiocytosis (Letterer-Siwe type) patient.

METHODS AND MATERIALS

Several 4-mm punch biopsies were removed under anesthesia from apparently healthy skin of the mid-volar forearm of an adult Caucasian and from the trunk and upper extremity of an infant with histiocytosis (Letterer-Siwe type). Following removal, biopsy specimens were placed in a solution of $\text{OsO}_4\text{-ZnI}_2$. This solution is a modification of that used by Maillet (13) and is prepared in the following way. 5 g of metallic iodine are mixed with 10-15 g of metallic zinc. The combined powders are slowly added to 200 ml of distilled water in a beaker (since this is an exothermic reaction, caution has to be observed during this step). After about 5 min the

solution is filtered. The final solution is prepared by combining 8 ml of the filtered ZnI_2 solution with 2 ml of unbuffered 2% OsO_4 . The OsO_4 - ZnI_2 fixative solution is kept in the dark before and during use.

The biopsy specimens were held in the OsO_4 - ZnI_2 solution for 24 hr at room temperature in darkness. They were then rinsed in distilled water and processed for embedding in Epon 812 (12). Thick sections (2–3 μ) were cut with glass knives on a Porter-Blum MT-1 ultramicrotome and were examined under the light microscope without staining. Thin sections in the grey to pale gold interference range (18) were cut with diamond knives on a Porter-Blum MT-1 ultramicrotome and mounted on Formvar-covered grids. Some sections were counterstained with 5% aqueous uranyl acetate solution (9) and Venable's lead citrate solution (20), while the majority were examined without counterstain. Pictures were taken with a Zeiss EM9A electron microscope operating at an accelerating voltage of 67 kv with a 50 $m\mu$ aperture in the objective lens.

Specimens serving as controls to OsO_4 - ZnI_2 were fixed in an osmium solution which had the identical OsO_4 concentration as the OsO_4 - ZnI_2 and the identical osmolarity but without the ZnI_2 (0.4% OsO_4 , pH 5.9, and 218 milliosmols). After fixation, this tissue was prepared for electron microscopic investigation exactly the same way as after OsO_4 - ZnI_2 fixation.

In order to elaborate on Maillet's assumption (14) that the OsO_4 - ZnI_2 -positive material may be lipid in nature the following organic solvents were used prior to the same prolonged fixation with OsO_4 - ZnI_2 : (a) 2:1 methanol/chloroform mixture at 60°C for 1 hr; (b) 70% methyl alcohol at room temperature for 1 hr; (c) primary fixation with 3% glutaraldehyde, followed by extraction with a series of graded alcohols and finally propylene oxide. The tissues were then sent through these solvents in reversed order, rehydrated, and fixed with OsO_4 - ZnI_2 for 24 hr at room temperature.

For routine electron microscopy, biopsy specimens from normal skin and skin of histiocytosis-affected skin were fixed at 4°C for 2 hr with distilled 3% glutaraldehyde (7, 8) in 0.1 M monosodium phosphate buffer pH 7.3. Following this primary fixation the tissue was washed in 0.1 M monosodium phosphate buffer, pH 7.3, for 2 hr. Secondary fixation was carried out at 4°C for 1 hr with 1% OsO_4 in 0.1 M monosodium phosphate buffer, pH 7.3. Sucrose was added to both the buffer wash and the secondary fixative so that the osmolar concentration of these solutions would be the same as that of the primary fixative (approximately 480 milliosmols).

RESULTS

Observations of Glutaraldehyde Osmium Tetroxide-Fixed Tissue

NORMAL EPIDERMIS: Langerhans cells can be recognized readily in the middle and upper epidermis by the presence of typical rod- and racquet-shaped organelles, the Langerhans cell granules (LCG) (19) (Figs. 1 *a* and *b*). The length of the LCG ranges from 100 $m\mu$ to over 1 μ . Their diameter varies from approximately 380 to 460 A. In longitudinal sections the LCG's are characterized by two limiting membranes, each approximately 120 A thick with a trilaminar substructure. Between these two limiting membranes and running parallel to them is a lamellar structure which may appear homogeneous or exhibit a distinct linear periodicity of approximately 90 A, depending upon the plane of section. This lamella bisects a moderately electron-opaque matrix and is separated from the aforementioned membranes. When LCG's are cut *en face*, they appear round or oval with a cross-striated pattern.

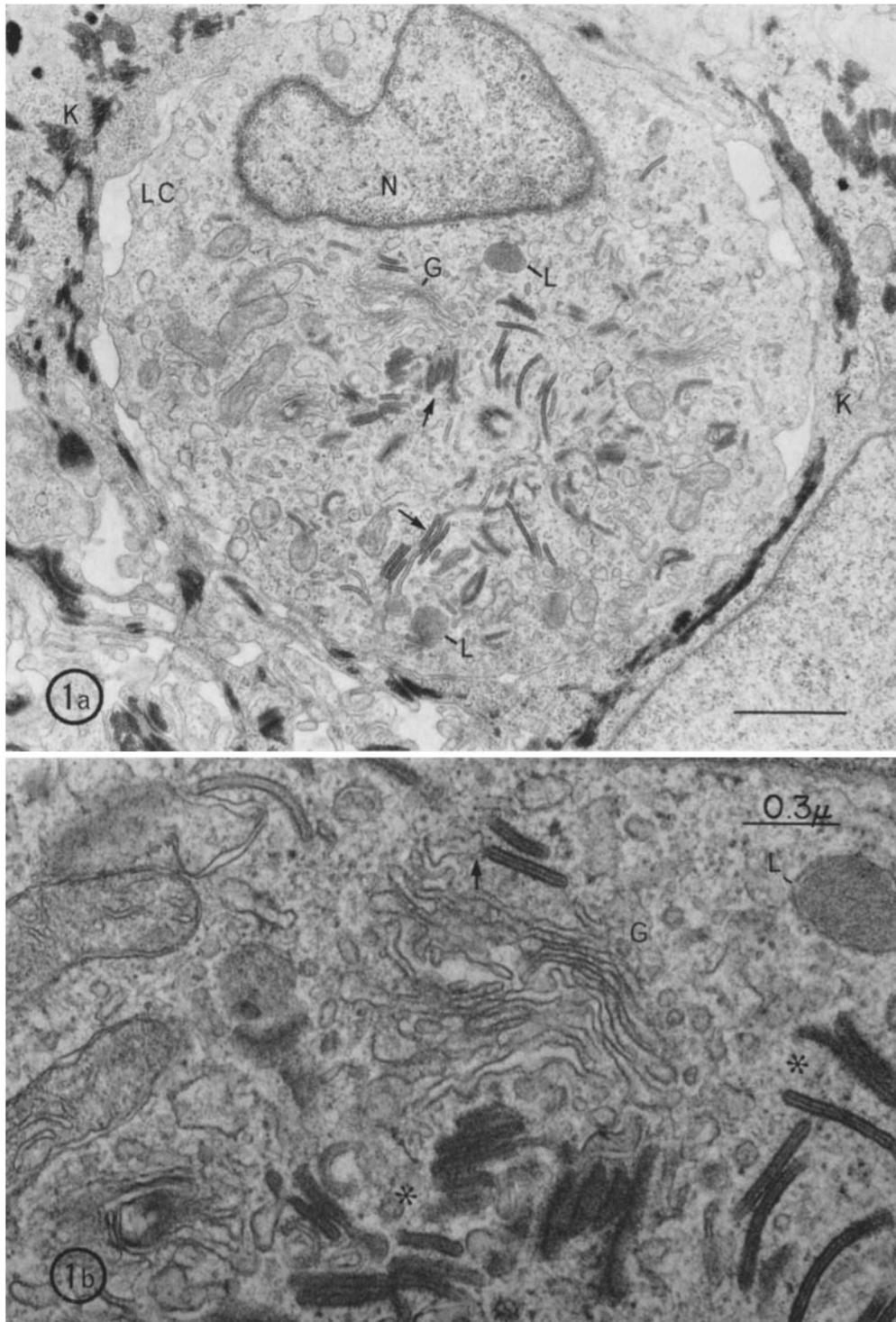
Large numbers of LCG's are found in proximity to the Golgi apparatus (Fig. 1). In the periphery of the cytoplasm the number of LCG's decreases. Occasionally granules attached to the cell membrane can be seen. In such instances, the limiting membranes of the granule are in direct continuity with the cell membrane, while the internal periodic lamella of the granule communicates directly with the extracellular space.

EPIDERMIS IN HISTIOCYTOSIS (LETTERER-SIWE DISEASE): Many Langerhans cells are easily recognized. In their substructure the LCG's in Letterer-Siwe disease are identical with the granules of normal epidermis, but their size and shape are more variable. Their proximity to the Golgi apparatus as seen in normal skin is less obvious. There is greater accumulation of LCG's at the periphery of the cell, and many of them are attached to the cell membrane (Figs. 2 *a* and *b*). At times the limiting membranes of the rod-shaped granule twist before continuing with the cell membrane (Fig. 2 *a*).

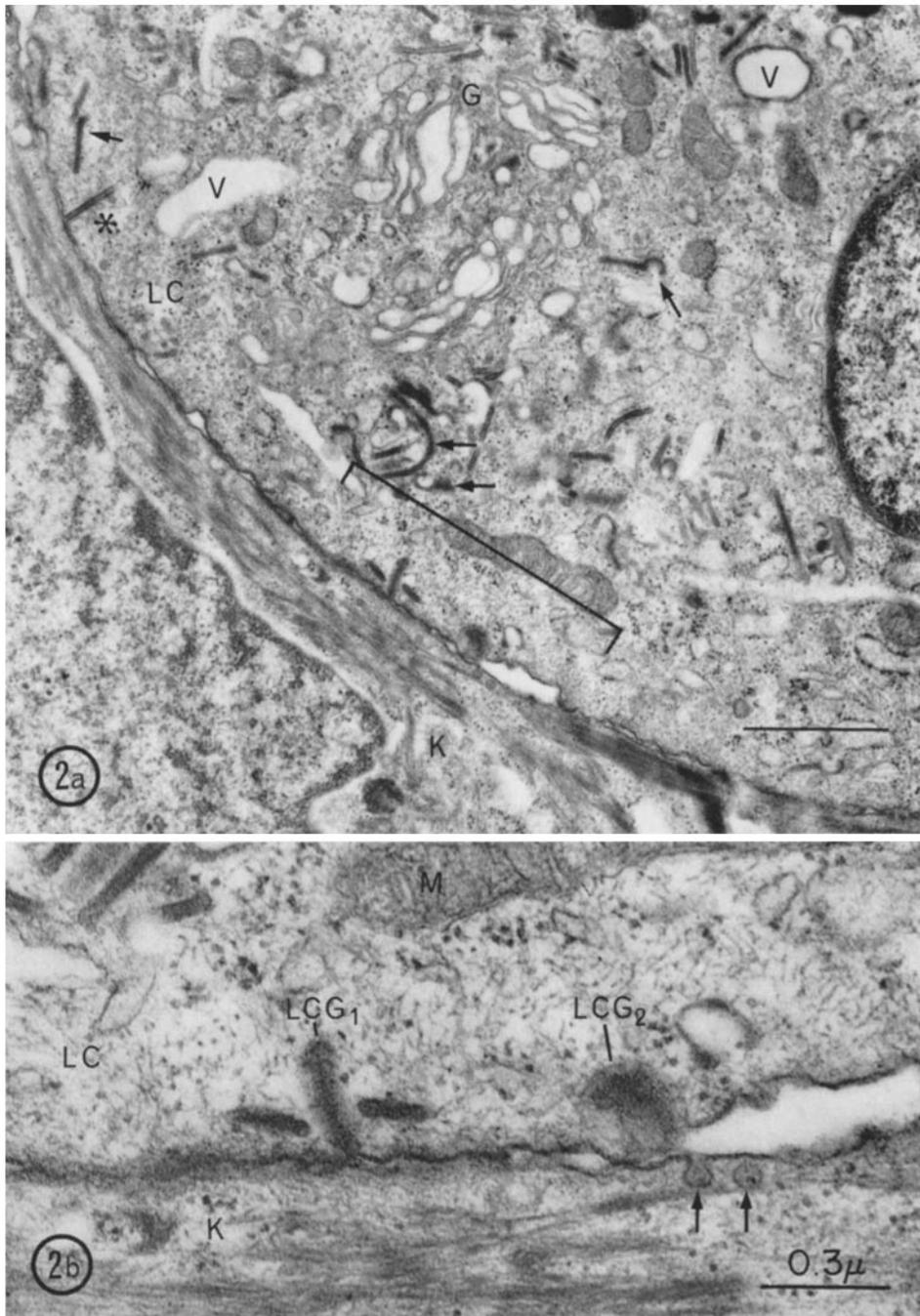
Observations of Osmium-Zinc Iodide-Fixed Tissue

LIGHT MICROSCOPY

In sections approximately 2 μ thick, the osmophilic dendritic cells of the epidermis are distinctly



FIGURES 1 *a* and *b* Normal human skin. Glutaraldehyde/OsO₄ fixation. Langerhans cell in suprabasal layers of epidermis. *a*, The Langerhans cell (LC) is characterized by desmosome-free plasma membrane, absence of tonofilaments from cytoplasm, indented nucleus (N), well-developed Golgi apparatus (G), centriole, and groups of rod-shaped LCG's (arrows). Note large number of LCG's in proximity to the Golgi area. L, lysosome-like bodies; K, keratinocytes. $\times 15,600$. *b*, Enlargement of the Golgi area (G) in Fig. 1 *a*. Groups of LCG's are marked by asterisks (*). Note proximity of LCG to smooth membrane of Golgi apparatus (arrow). L, lysosome-like body. Scale lines equal 0.3μ . $\times 46,800$.



FIGURES 2 *a* and *b* Skin of Letterer-Siwe disease. Glutaraldehyde/OsO₄ fixation. Langerhans cell in supra-basal layers of epidermis. *a*, The Golgi apparatus (*G*) of Langerhans cell (*LC*) is well developed. A large number of rod-shaped and racquet-shaped profiles of LCG's (arrows) are located in the periphery of the cell. Some are attached to the plasma membrane(*). Large membrane-limited vacuoles are present (*V*). *K*, keratinocyte. $\times 18,300$. *b*, Enlargement of the bracketed area in Fig. 2 *a*. Rod-shaped profile of LCG's (*LCG*₁) and LCG cut *en face* (*LCG*₂) attached to the plasma membrane of Langerhans cell (*LC*). *LCG*₂ appears as a cross-striated disc and is partly localized in the extracellular space. The plasma membrane of the adjacent keratinocyte (*K*) contains pinocytotic vesicles (arrows). *M*, mitochondrion. Scale lines equal 0.3 μ . $\times 46,800$.

demonstrated and easily differentiated from keratinocytes which stain far less. Figs. 3 *a* and *b* are two light micrographs, in different focal planes, of a section without counterstain, showing the selective deposition of metal and the dendritic nature of the Langerhans cell.

ELECTRON MICROSCOPY

NORMAL EPIDERMIS: The fine structure of the tissue is preserved well enough after a 24 hr exposure to the $\text{OsO}_4\text{-ZnI}_2$ mixture, but at high magnification fine details may be blurred (depending upon amount of metal deposited) and artificial spaces can be seen between the dendritic cells and the neighboring keratinocytes. The striking electron-opaque appearance of the Langerhans cell (Figs. 4, 5) is produced by heavy deposition of metal on the nuclear envelope, the Golgi apparatus, some of the lysosomal structures, and in particular the LCG's. There is, however, no heavy metal deposition on the cell membrane or the trilaminar structure of intracytoplasmic membranes which nevertheless can faintly be recognized.

The morphology of the LCG as revealed by the $\text{OsO}_4\text{-ZnI}_2$ method in longitudinal, oblique, and tangential planes can be observed in more detail in Fig. 5 *b*. Longitudinal sections of the LCG's are easily recognized by the intense reaction of a

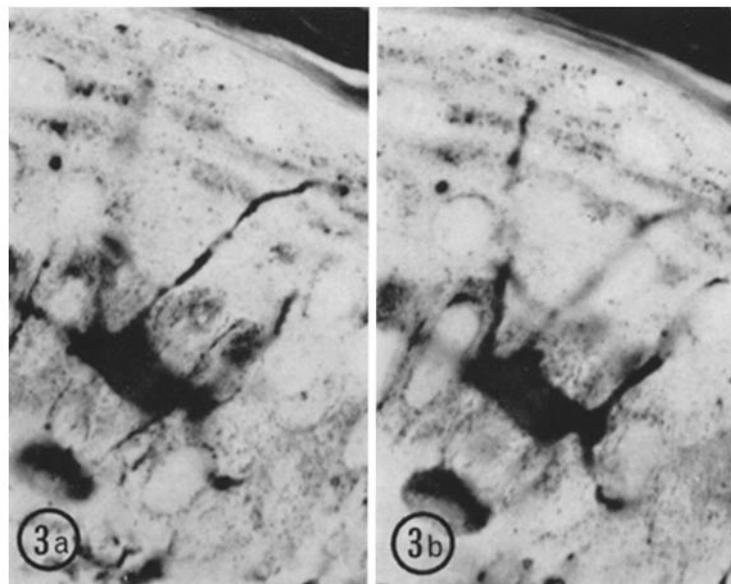
centrally positioned, striated lamella, whereas the limiting membranes and the homogeneous matrix do not stain. In the racquet-shaped profiles, the staining material of the central lamella continues along the inner surface of the vesicular dilatation where it still exhibits its characteristic periodicity (Fig. 5 *b*). After observing many sections cut from different blocks, we have not seen metal deposition on LCG's which have fused with the cell membrane (Figs. 4, 5 *b* and *d*).

The Golgi apparatus is entirely impregnated, and the deposits of the reaction product are localized within the lumen and along the inner surface of the cisternae, in many of the vesicles at the ends of such cisternae, and in the vesicles and vacuoles associated with the Golgi complex (Figs. 4, 5). Most of the metallic deposits are totally electron-opaque. The proximity between the metal deposition in LCG's and that in the Golgi complex is strikingly evident.

The nuclear envelope regularly shows heavy metal deposition. The cytoplasmic surface of the envelope is rough due to the adherence of numerous small electron-opaque particles.

Many of the lysosome-like bodies exhibit varying degrees of electron opacity; some of these organelles are almost completely electron-opaque (Fig. 5).

EPIDERMIS IN HISTIOCYTOSIS (LETTERER-



FIGURES 3 *a* and *b* Normal human skin after fixation with $\text{OsO}_4\text{-ZnI}_2$ mixture and without counterstain. Two light micrographs of 3- μ sections in two different focal planes. $\times 1,600$.

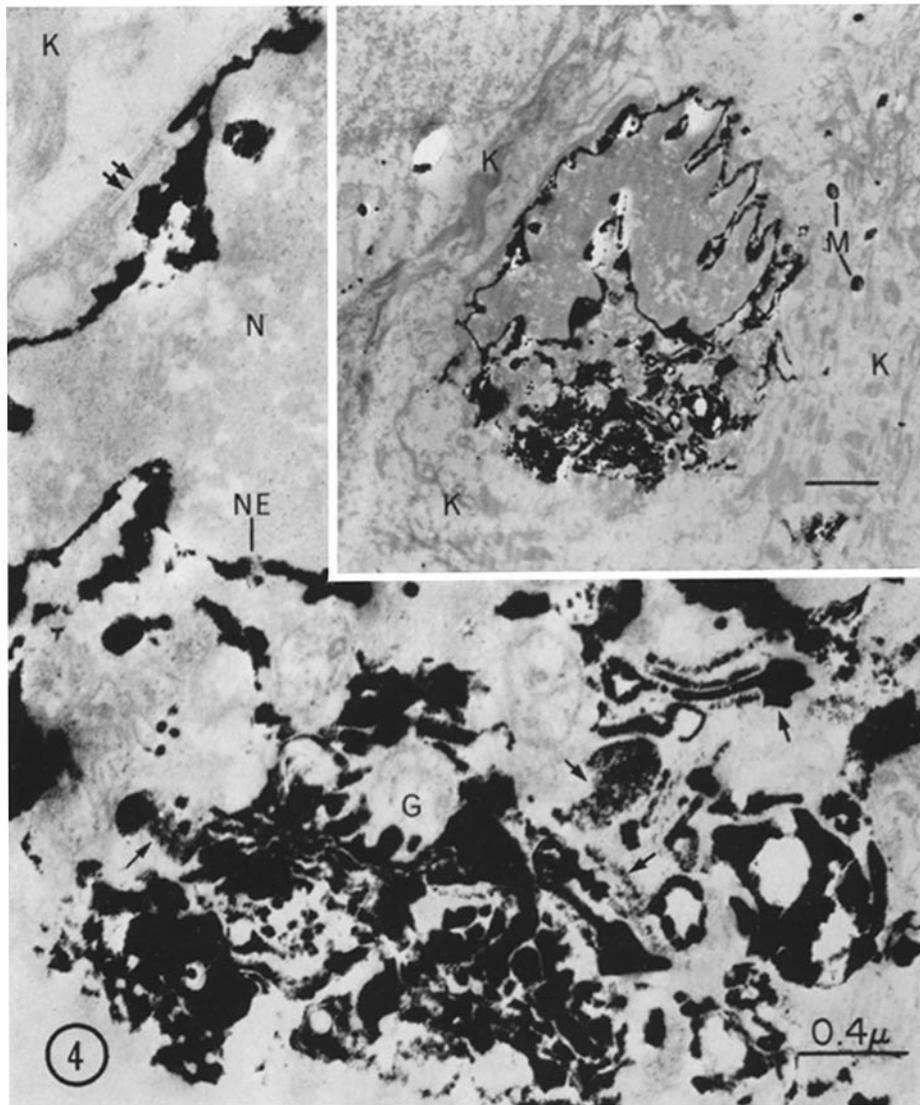
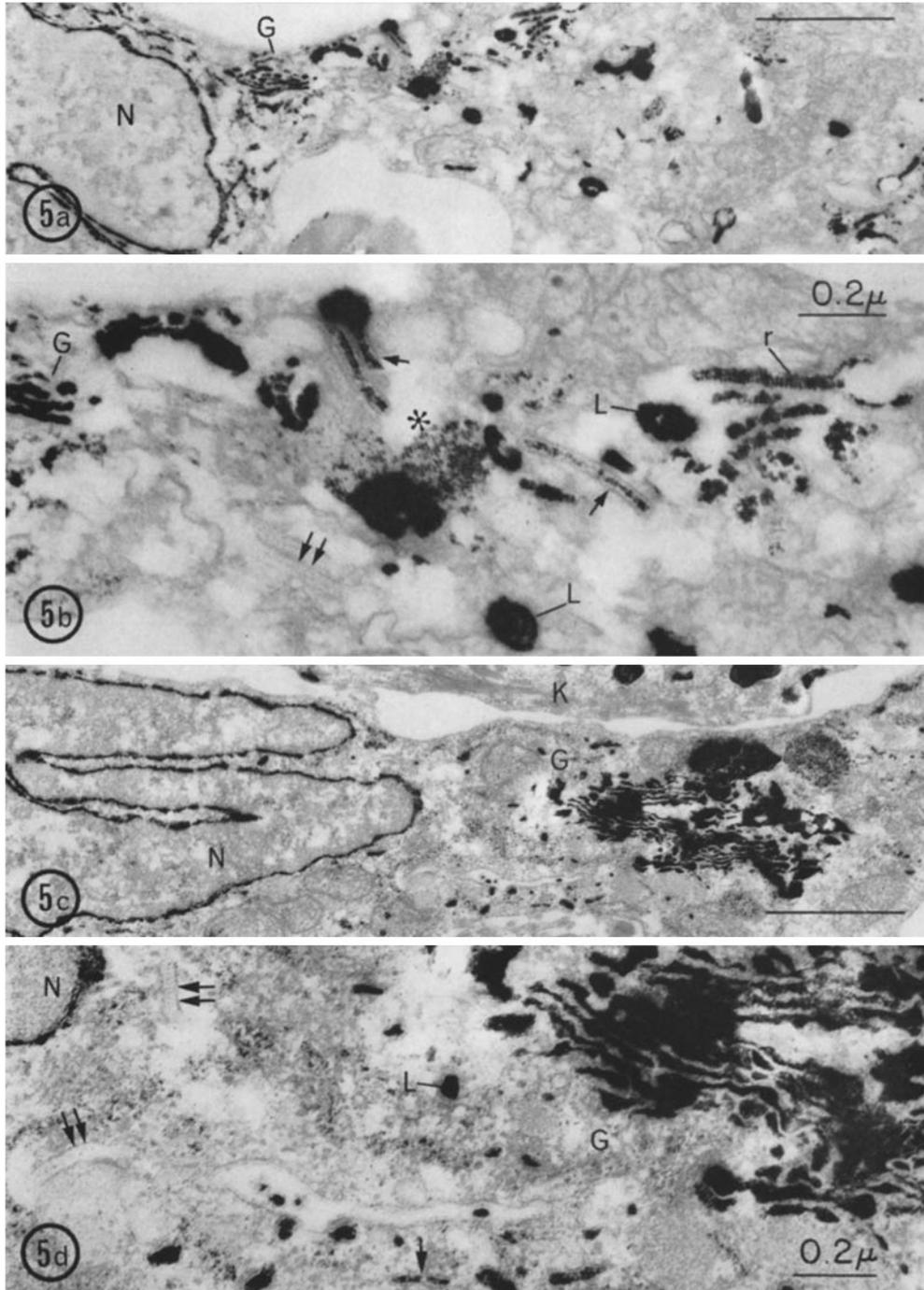


FIGURE 4 Normal human skin. $\text{OsO}_4\text{-ZnI}_2$ fixation. Langerhans cell in suprabasal layers of epidermis. The cisternae and vesicles of the Golgi apparatus (*G*), the nuclear envelope (*NE*), and the LCG's (arrows) exhibit dense deposits of metal. The LCG's appear as striated rods in longitudinal sections or as cross-striated discs when cut *en face*. Note that the plasma membrane and rod-shaped LCG in the periphery of the cell (double arrow) do not stain. *N*, nucleus. $\times 35,150$. Inset: Low-magnification electron micrograph of the same area. The heavily impregnated Langerhans cell can be differentiated from the keratinocytes (*K*) in which only melanosomes (*M*) exhibit metal deposition. Scale lines equal 0.4μ . $\times 10,200$.

SIWE DISEASE): Unlike the LCG's of the normal epidermis, the granules in Langerhans cells in Letterer-Siwe disease are found in larger numbers in the periphery of the cell. Again, granules attached to the plasma membrane do not stain (Figs. 6, 7). With regard to their substructure

and staining property, the LCG's fixed with $\text{OsO}_4\text{-ZnI}_2$ are identical with those just described in normal skin. The Golgi region and the nuclear envelope show the same degree of metal deposition as is seen in Langerhans cells of normal epidermis. Lysosome-like bodies also exhibit a strong reac-



FIGURES 5 a-d Normal human skin. OsO₄-ZnI₂ fixation. Two different Langerhans cells in suprabasal layers of epidermis. The nuclear envelope (N, nucleus), the Golgi apparatus (G), and the LCG's (arrows) exhibit dense metal deposits. K, keratinocyte. a, $\times 19,500$. b, Enlargement of area adjacent to the Golgi apparatus in Fig. 5 a. In rod-shaped profiles (arrows) and racquet-shaped profiles (r) the striated central lamella exhibits an intense reaction, while the limiting membranes do not stain. The asterisk (*) denotes LCG *en face*. In the vesicular portion of the LCG (r) the metal deposit also exhibits a periodicity. Note unstained LCG (*en face*) (double arrow). L, lysosome-like bodies. $\times 58,500$. c, $\times 19,500$. d, Enlargement of area adjacent to the Golgi apparatus in Fig. 5 c. Note stained LCG (arrow) and unstained LCG (double arrows). Scale lines equal 0.2μ . $\times 58,500$.

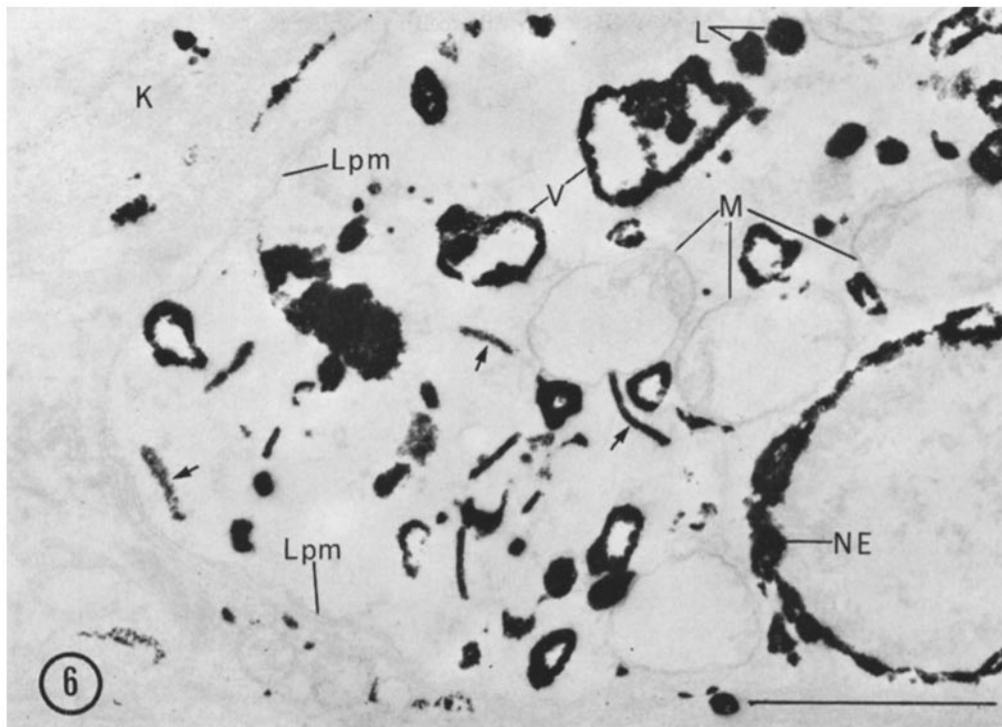


FIGURE 6 Skin of Letterer-Siwe disease. $\text{OsO}_4\text{-ZnI}_2$ fixation. Langerhans cell in suprabasal layers of epidermis. The nuclear envelope (NE), lysosome-like bodies (L), rod-shaped LCG's (arrows), and the periphery of several large vacuoles (V) exhibit dense deposits of metal. The plasma membrane of the Langerhans cell (Lpm) does not stain. M, mitochondria; K, keratinocyte. $\times 33,000$. Scale line = 1μ .

tion and appear almost electron-opaque (Fig. 6). The striking distinction between Langerhans cells of normal epidermis and those of epidermis in Letterer-Siwe disease is the presence of large vacuoles (Fig. 6). The following two types of large, membrane-limited vacuoles can be distinguished: those reacting with $\text{OsO}_4\text{-ZnI}_2$ and showing heavily impregnated peripheries, and those without any deposition of metal (Fig. 7).

CONTROLS: Control biopsy specimens fixed with a solution containing only 0.4% osmium tetroxide for 24 hr were examined at both the light and electron microscopic levels. No heavy deposition of metal was evident.

After treatment of biopsy specimens with lipid solvents prior to exposure to $\text{OsO}_4\text{-ZnI}_2$, no evidence of heavy osmium deposition could be observed, although the tissues were still well enough preserved to be recognized by their background stain.

DISCUSSION

The results of this technique represent the first successful staining of the LCGs. So far, all histochemical attempts to demonstrate the LCG's have failed (1, 23). Neither the gold chloride technique nor the ATPase method made it possible to obtain selective staining of the LCG's (26). The osmium iodide method, introduced by Champy in 1913 (5) for the study of neural elements, was tried with little success (2). To standardize the Champy technique, Maillet studied miscellaneous agents and proposed a new reaction product consisting of OsO_4 and ZnI_2 (13). This technique consistently demonstrated epidermal dendritic cells in light microscopic preparations (15).

Electron microscope examination of $\text{OsO}_4\text{-ZnI}_2$ -fixed Langerhans cells reveals that the centrally positioned lamella of the organelle displays a striking periodic impregnation, whereas the limit-

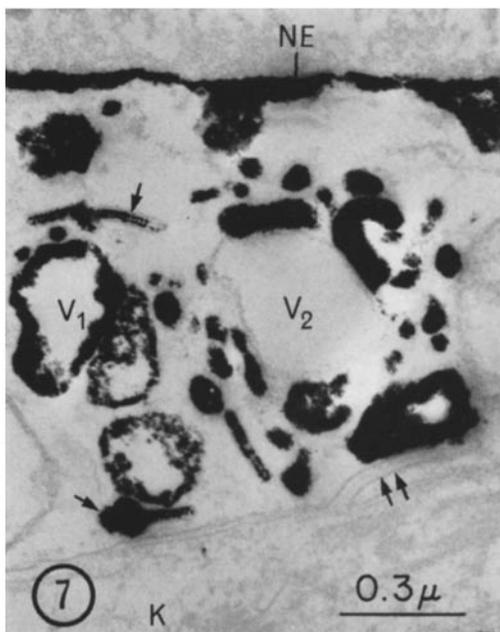


FIGURE 7 Skin of Letterer-Siwe disease. $\text{OsO}_4\text{-ZnI}_2$ -fixation. Langerhans cell in suprabasal layers of epidermis. The nuclear envelope (NE), the striated central lamella in the rod-shaped LCG's (arrow), and the periphery of a large vacuole (V_1) exhibit dense metal deposits. The other large vacuole (V_2) does not show any deposition of metal. Note that the plasma membrane and attached LCG (double arrow) do not stain. K, keratinocyte. Scale line equals 0.3μ . $\times 54,000$.

ing membranes and the matrix remain unstained.

After $\text{OsO}_4\text{-ZnI}_2$ -fixation, LCG's become better visualized and more frequently visible than LCG's observed in routinely fixed tissue. While a majority of the LCG's located near the Golgi region show the preferential deposition of metal, most LCG's at the cell periphery remain unstained both in normal epidermis and in Letterer-Siwe diseased epidermis. The differences in the staining property exhibited by LCG's near the Golgi region and by those at the cell periphery, respectively, may be the result of structural and/or metabolic changes of the granules involved.

One group of investigators working on normal epidermis believes that LCG's are synthesized at the Golgi apparatus (25). Another group working on skin in Letterer-Siwe disease believes that these granules are formed by endocytotic infoldings of

the cell membrane (10). Both groups of workers presumably formulated their opinions on the basis of the location where they saw LCG's most strikingly. Our investigations have shown that the prevalent localization of LCG's differs; it has been near the Golgi apparatus in normal epidermis, and near the plasma membrane in Letterer-Siwe disease epidermis. Thus, the different localizations of LCG's in Langerhans cells of normal epidermis and of epidermis in Letterer-Siwe disease have led, as we believe, to different interpretations. Our results seem to favor the assumption that there may be a close relation between the Golgi apparatus and the LCG's. We believe that these granules do not arise from the cell membrane, because the granules have an electron-opaque periodic and complex structure and because we have not been able to demonstrate any localized electron opacity on the cell membrane such as is seen in the LCG's.

The chemical nature of the material which is preferentially and heavily impregnated with metal by the $\text{OsO}_4\text{-ZnI}_2$ technique cannot be ascertained at present. Extraction of the tissue with lipid solvents abolished all heavy metal impregnation but left sufficient background stain for tissue recognition. Among the many substances that could be extracted with the aforementioned solvents, lipids are considered the primary ones. This assumption corroborates the work of Maillet (14) who postulated that the $\text{OsO}_4\text{-ZnI}_2$ acts to uncouple lipid moieties from lipoprotein complexes and that newly exposed groups of the lipid would then be available for increased deposition of metal.

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