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TRANSFER MECHANISM OF MELANOSOMES IN EPIDERMAL CELL CULTURE

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The mode of melanosome transfer from melanocytes to keratinocytes in epidermal cell cultures has been examined with time-lapse cinematography and electron microscopy. A tip of a melanocyte dendrite containing melanosomes became enfolded by a recipient keratinocyte. It was then pinched off to form a cluster of melanosomes which initially seemed to be surrounded by two layers of membranes. The phagocytized dendrite was gradually decomposed and became an aggregate of melanosomes surrounded by a single membrane of the keratinocyte. The individual melanosomes were dispersed from the aggregate into the keratinocyte cytoplasm, depending on the size of melanosomes. The larger ones were single and smaller ones were complex.

The mechanism of melanosome transfer in vitro is a type of cytophagocytosis. The entire process consists of two steps: the first is a cytophagic process and the second a melanosome dispersion process. The process is influenced by various exogenous factors.

Melanin pigmentation of the skin results from the close interaction between epidermal melanocytes and keratinocytes. Melanosomes, which are the specific cytoplasmic particles upon which melanin formation and deposition occur, are synthesized in the melanocytes (melanogenesis). The melanosomes are transferred to basal keratinocytes (melanosome transfer). Transferred melanosomes disperse within the cytoplasm of the keratinocytes (dispersion of melanosomes). Basal cells containing melanosomes move upward continuously to become spinous and finally horny cells during which time melanosomes disintegrate to some extent (turnover of keratinocytes and degradation of melanosomes is phagocytized by the keratinocyte somes from melanocyte to keratinocyte constitutes an important factor in skin melanin pigmentation.

The transfer mechanisms have been studied with light and electron microscopy in epidermis [1,2], hair [3-5], and cell culture systems [6-12]. It is now generally assumed that the transfer of melanosomes occurs directly from cell to cell and a portion of the melanocyte dendrite containing melanosomes is phagocytized by the keratinocyte [3,6,7,9-11,13]. Melanosomes, thus transferred, disperse within the cytoplasm of the keratinocyte [2,8,14,15]. These observations were made either with time-lapse cinematography on cultured cells or electron microscopy of epidermis and hair. The exact sequences of the cytophagocytotic process still need to be clarified and the data reported in this paper were aimed at this clarification. It is known that melanosomes distribute ultimately in the keratinocytes in two forms: as single, discrete particles, and as multiple, aggregated particles in melanosome complexes [13,15-18]. Recent observations have led to the hypothesis that these different patterns may be related to the size of the individual melanosomes [1,2,5,14-16,19].

In the present paper, we have studied the dispersion of melanosomes, which had been transferred as a cluster, within the cytoplasm. The results indicate that melanosomes separate off from the aggregate of melanosomes singly or as a group of particles, depending on their sizes.

MATERIALS AND METHODS

Cell culture. Small skin specimens were obtained from newborn or adult black guinea-pig ears. The specimens were rinsed thoroughly with Tyrode's solution containing penicillin (1000 units/ml) and streptomycin (1 mg/ml), and then with calcium-free and magnesium-free Tyrode's solution (CMF). They were incubated in CMF for 15 min at 37°C, followed by 1% trypsin (Difco, 1:250) solution for 30 min at 37°C, and again with CMF. The epidermis was separated from the dermis and transferred in the culture medium to a depression slide, where it was squeezed, shaken, and pulled apart with a fine forceps. The cell suspension thus obtained was centrifuged at 1000 rpm for 5 min and the cells were resuspended in culture medium. They were cultured in Rose chambers or Falcon plastic Petri dishes at 37°C for several days, and the culture medium, which was Eagle's minimum essential medium supplemented with 20% fetal bovine serum, was changed every other day.

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Time-lapse cinematography. The cell behavior was recorded with time-lapse cinematography using both phase contrast and transmission light microscopy.

Scanning electron microscopy. The cells were grown on glass cover slips in 60-mm Falcon Petri dishes for scanning electron microscopy. They were fixed at appropriate times in 2.5% glutaraldehyde and 2% osmium tetroxide solution in 0.06 M veronal acetate buffer, pH 7.2, containing 4.5% sucrose for 20 min at 4°C. They were rapidly dehydrated with a graded series of ethanol, and the specimens thus obtained were infiltrated with gradually increasing concentrations of isoamylacetate in ethanol to replace the ethanol. Finally, they were dried by means of the carbon dioxide critical-point drying method using a Hitachi Critical Point Dryer (HCP-1). The specimens were mounted on a holder with silver-conducting paint and were coated with carbon and gold-palladium alloy in a Hitachi vacuum evaporator equipped with a rotary stage. The samples thus prepared were examined in a JEOL-50A scanning electron microscope.

Transmission electron microscopy. The cells were grown in 35-mm Falcon tissue culture dishes, of which the inside surface had been precoated with Epon 812. They were fixed as described above for 20 min at 4°C, briefly washed with 50% ethanol, counterstained with uranyl acetate solution for 10 min at 4°C, and subsequently dehydrated with a graded series of ethanol. They were infiltrated in 50% Epon-812 in ethanol for 30 min before final embedding. Thin sections were cut vertically to the surface of the Petri dish with an LKB Ultrotome III equipped with a diamond knife. Serial sectioning was performed when various stages of melanosome transfer were followed. The thin sections were examined with a Hitachi HU-12A electron microscope.

RESULTS

Light Microscopic Observations

By the end of a week the cultures became well established and melanocytes and keratinocytes were numerous. The keratinocytes were flattened and formed sheets, the melanocytes had multiple dendrites and a large number were associated with keratinocytes. Pigment donation was observed from the melanocytes to the keratinocytes. The processes began with contact between the actively ruffling cytoplasmic membranes of the keratinocytes and the tips of the branching processes of the melanocytes. The tip of a dendrite containing numerous melanosomes seemed to penetrate the keratinocyte, after which the dendrite appeared to be constricted and a small pouch formed.

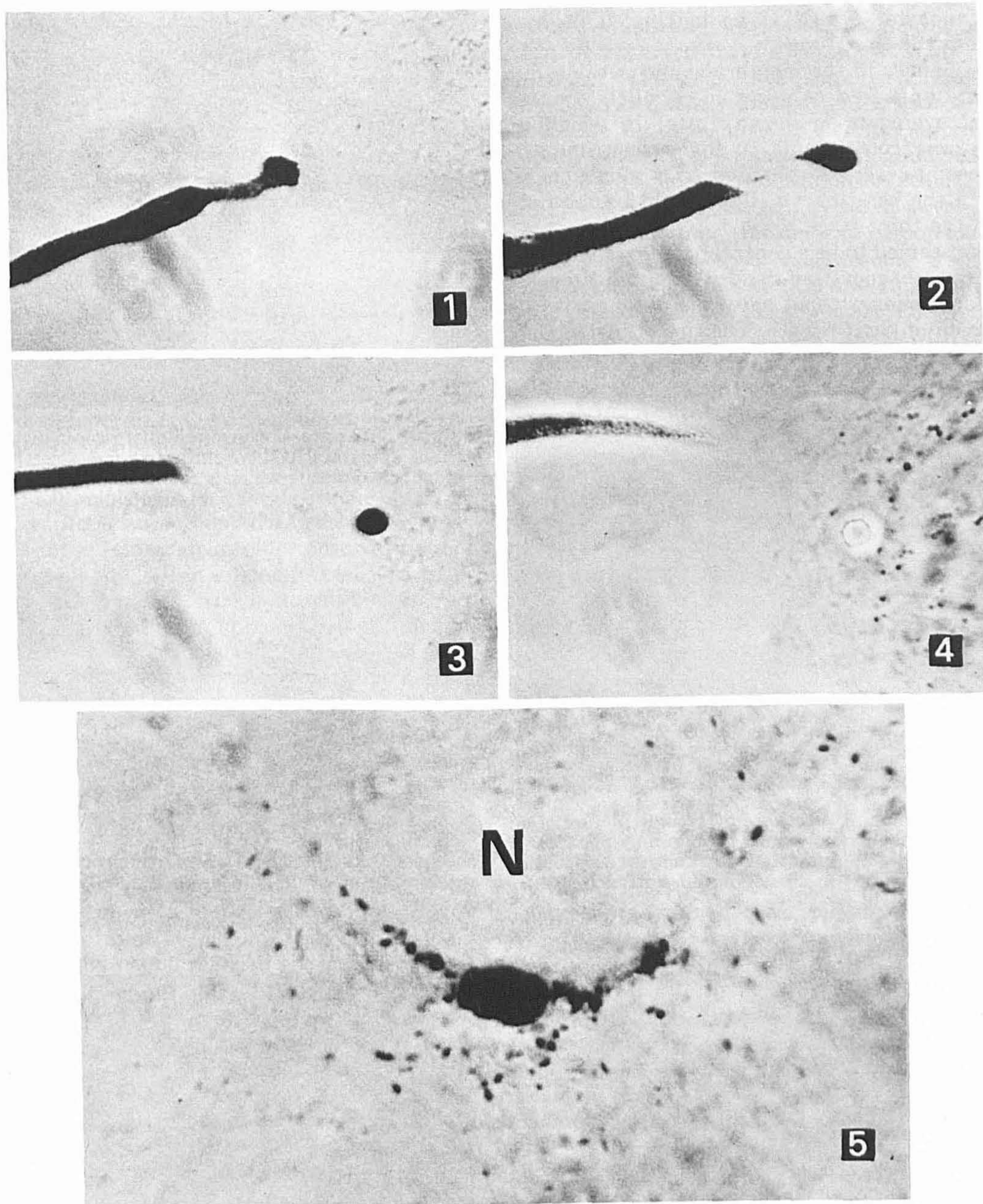
Figure 1 shows the initial stage of melanosome transfer under the usual transmission light microscope. Only melanosomes were visible so the behavior of melanosomes during the transfer process could easily be followed. One hour later, as shown in Figure 2, the constriction was almost completed, although the pouch was still connected by a fine string to the stem of the dendrite. The dendrite gradually began to retract, and 3 hr later the pouch appeared to be pinched off completely from the stem dendrite as shown in Figure 3. This pinching-off process seemed to result from both the constrictive movement of the keratinocyte and the withdrawal movement of the branching process of the melanocyte. Under the phase contrast microscope, as shown in Figure 4, the pouch phagocytized in the keratinocyte was in the cytoplasm, while the dendrite, the tip of which was pinched off, appeared to be withdrawn. The pouch, containing the phagocytized tip of the dendrite, gradually moved towards the perinuclear area where it tended to disperse singly or as a group of melanosomes, as shown in Figure 5. In newborn guinea-pig skin, the pouch was usually large, containing large numbers of melanosomes. Such large clusters of melanosomes disintegrated into several smaller clusters, then into single or groups of melanosomes. As shown in Figures 1, 2, and 3, melanosomes were transferred from the melanocyte to the keratinocyte as a cluster enclosed with the cytoplasmic membrane of the melanocyte dendrite, and melanosomes were never transferred individually from the dendrite of the melanocyte into the culture medium. Eventually, single melanosomes or groups of a few melanosomes were found to be dispersed around the perinuclear area of the keratinocyte.

Scanning Electron Microscopic Observations

The cellular interaction between melanocyte and keratinocyte was observed more clearly under the scanning electron microscope. Figure 6 shows one of the typical views of the epidermal cell cultures obtained from newborn guinea-pig skin. One melanocyte with two long, slender dendrites and a shorter one was in contact with two keratinocytes through the tips of the dendrites. The tip of the dendrite seen on the left side had been introduced into the cytoplasm of the keratinocyte and seemed to be constricted. The cytoplasm of the melanocyte was filled with uniform small particles which corresponded to individual melanosomes. The margins of the cytoplasm of these cells were clearly seen so that the relationship between these melanocyte dendrites and the keratinocyte cytoplasm could be defined more precisely. Figure 7 shows at higher magnification one of the tips of the dendrites seen in Figure 6. The dendrite, filled with many melanosomes, appeared to be at the constricted stage since a fine filamentous structure was seen between the tip and the stem. On the other hand, a few clusters of melanosomes and scattered individual melanosomes could be seen in the cytoplasm.

Transmission Electron Microscopic Observations

Various steps of melanosome transfer from the melanocyte to the keratinocyte were observed in more detail under the electron microscope in vertical sections of the cultures. Figure 8 shows a tip of a dendrite which had penetrated into a keratinocyte, and had become enfolded with the cytoplasmic membrane and with several villus-like cytoplasmic projections of the keratinocyte. This corresponds to the first stage of the melanosome transfer process. Figure 9 shows a typical electron



FIGS. 1–5. The melanosome transfer processes between melanocytes and keratinocytes in guinea-pig skin culture. *1:* A. melanocyte dendrite is introduced into a keratinocyte (× 600). *2:* The dendrite is constricted by the keratinocyte (× 600). *3:* The tip of the dendrite is torn off and hauled in close to the nucleus of the keratinocyte to form a cluster of melanosomes (× 600). *4:* Corresponding to Fig. 3, but taken with a phase contrast microscope. The resulted cluster of melanosomes is located near the nucleus of the keratinocyte (× 600). *5:* Melanosomes are dispersing from the cluster of melanosomes located near nucleus (× 1000).

micrograph of the cultured cells obtained from adult guinea-pig ear. The horizontal line indicates the surface of the Falcon plastic dish on which cells had grown. Several keratinocytes were piled up in layers and one of these contained several groups of melanosomes. As shown in Figure 10 at higher magnification, one of these groups of melanosomes seemed surrounded by two concentric layers of membranes. In the package, cytoplasmic constituents of the melanocyte, such as free ribosomes for the outer membranes of individual melanosomes, were seen to be intact. This could represent the second stage of the transfer process, in which the tip of dendritic process was phagocytized by the keratinocyte. In other areas (*b*), the outer membrane seemed to be intact, but the inner one and the outer membranes of individual melanosomes were vague, and the other cytoplasmic constituents

of melanocyte seemed to be lacking. This may represent the third stage.

The changes in the membrane and cytoplasmic elements appeared to occur in parallel. Another type of aggregate is shown at (c) in which no cytoplasmic constituents could be seen and the melanosomes were surrounded by a single membrane of the keratinocyte. This could be the final step of the third stage.

There seemed to be a general pattern of enfolded dendrites, phagocytized dendrites, and aggregates of melanosomes without outer membranes, in the cytoplasm of keratinocyte. The tips of melanocyte dendrites were located in the peripheral region of

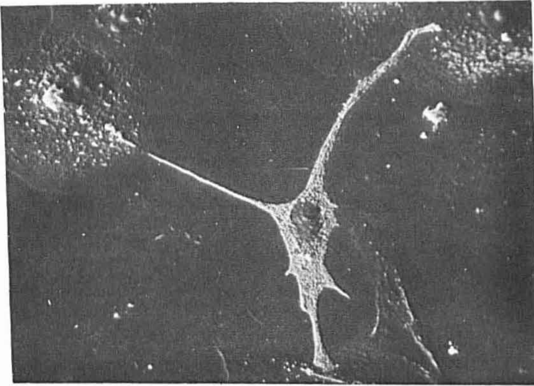


FIG. 6. A scanning electron microscopic picture of an epidermal cell culture obtained from guinea-pig skin. A melanocyte at the center is in contact with the surrounding keratinocytes by two slender, long dendrites (× 330).

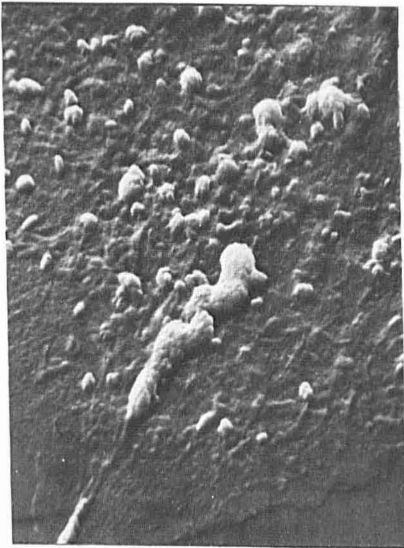


FIG. 7. Higher magnification of a part of Fig. 6. The tip of the dendrite in which numerous melanosomes are accumulated is found to be constricted but there still remains a thin string between the stem and the tip of the dendrite. A few clusters of granules and several scattered granules are seen far from the tip of the dendrite (× 1,900).

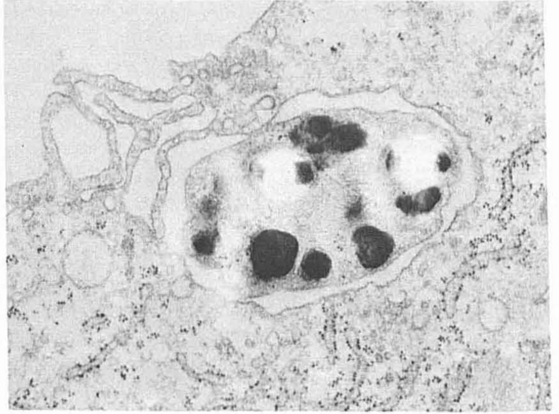


FIG. 8. Transmission electron microscopic picture of a part of epidermal cell in culture. A cross-section of a melanocyte dendrite which is enfolded with keratinocytic plasma membrane and several microvillus-like cytoplasmic projections (× 14,000).

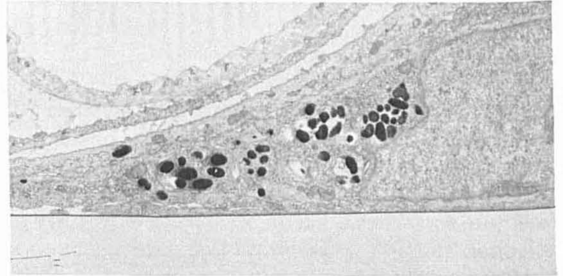


FIG. 9. A vertical section of epidermal cell culture obtained from adult guinea-pig skin. Several keratinocytes are piled up in layers, and one of these cells holds various types of groups of melanosomes (× 4,300).

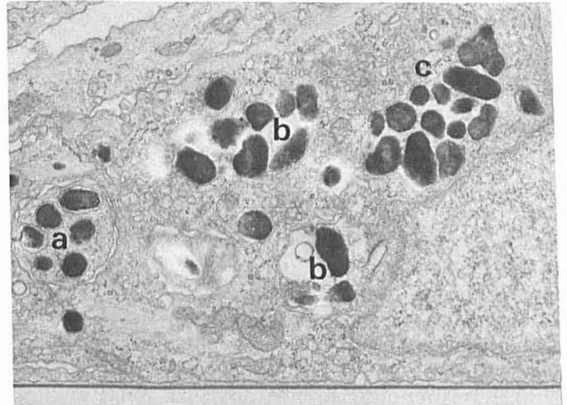


FIG. 10. Higher magnification of a part of Fig. 9 (× 12,500). *a:* A group surrounded by two layers of membranes. *b:* A group surrounded by one intact outer membrane and one diminishing inner membrane. *c:* Aggregates surrounded by an apparently single membrane.

keratinocyte; the phagocytized dendrites, in which the digestion of cytoplasmic contituents of melanocyte was in progress, were present between the nucleus and the periphery; the aggregates of mel-

anosomes surrounded by a single membrane were located near the nucleus. Figure 11 shows another view of a cell culture of newborn guinea-pig epidermis. This cell contains single, discrete melanosomes; groups of several melanosomes; and a huge aggregate of melanosomes surrounded by a single membrane. Such a huge aggregate is seen frequently in cell cultures derived from newborn animals. In this mass, the single envelope membrane is invaginated in various directions and the large clump is disintegrating into several smaller pieces. On the other hand, there were many single melanosomes and several groups of melanosomes in the vicinity of this aggregate. They were scattered from the aggregate and there was a tendency for small melanosomes to be complexed and for the large ones to be dispersed singly.

DISCUSSION

Phase-contrast microscopy has been widely used for time-lapse cinematographic observations of cultured cells including pigment transfer studies [5,6,8]. Under the phase-contrast microscope, individual melanosomes are difficult to distinguish from other cytoplasmic particles. Transmission light microscopy was used extensively in this series of time-lapse cinematographic observations on the interaction between melanocytes and keratinocytes in culture. As shown in Figures 1, 2, 3, and 5, the behavior of individual melanosomes could be observed clearly throughout the transfer processes. Individual melanosomes were never released from the dendritic processes even during the penetration and the pinching-off stages. They appeared to separate off individually or as a group from the pouch filled with melanosomes after the pouch reached the nucleus of the keratinocyte. The results showed definitely that the melanosome transfer process in vitro is, as has been inferred, cytophagocytosis. These observations were confirmed by the results obtained with the scanning electron microscope, in which the surface structures of the cells and the interaction

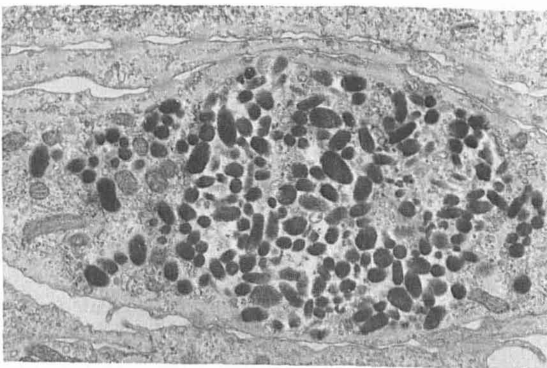


FIG. 11. A huge aggregate of melanosomes is seen in a cultured keratinocyte of newborn guinea-pig skin. The envelop membrane of this aggregate is seen to be invaginated and small packages of melanosomes are also seen near the aggregate (× 8,800).

between dendritic processes and keratinocytes could be observed in more detail at higher magnification. The cells in culture were fixed and embedded frequently during light microscopic observations for transmission electron microscopic studies, so that the dynamic events could be linked with the static ultrastructural observations. Serial thin sections were made in order to observe various steps of melanosome transfer and dispersion.

The very first step of melanosome transfer is represented by penetration of the tip of the dendritic process into the keratinocyte. The tip of the dendrite, as shown in Figure 8, infolds with the cell membrane and the villus-like cytoplasmic projections of the keratinocytes. There is a wide gap between the dendritic process and the keratinocyte cytoplasm. The second step corresponds to the one shown in (*a*) marked in Figure 10 where the tip of dendritic process is pinched off and a cluster of melanosomes is embedded in a cytoplasmic matrix surrounded by two membranes: one derived from the melanocyte and one belonging to the keratinocyte. The gap is very narrow. These two findings, shown clearly in Figure 8 and (*a*) marked in Figure 10, are the most important evidence to support the hypothesis that melanosome transfer in vitro is carried out by the cytophagocytotic mechanism. In specimens in vivo, similar figures have been observed in hair bulb [3,5], developing fowl feathers [4], and epidermis [15]. Under the electron microscope, various types of masses of melanosomes, as seen in (*a*), (*b*), and (*c*) marked in Figure 10, and in Figure 11, were observed in the keratinocyte. They are assumed to show the digestive process of cell constituents derived from melanocyte dendrite. Although there is no evidence to show that such digestive processes proceed in the same general manner as in the lysosomal heterophagic vacuoles, these aggregates of melanosomes surrounded by a single membrane are thought to be a sort of lysosomal digestive vacuole. We avoid calling them melanosome complexes and call them aggregates of melanosomes (or pouches filled with melanosomes), since the term "melanosome complex" has generally been used to indicate that melanosomes themselves are subjected to digestive processes in heterophagic and/or autophagic vacuoles [13]. The melanosomes, except their outer membranes, do not appear to be affected during these digestive stages. These two stages, the phagocytized and the digestive stages of the dendritic processes of melanocyte, can be recognized in the hair bulbs of C3H mice in vivo. Therefore, the transfer mechanism of melanosomes should be, as has been inferred by others [3,6,7,9–11,13,20], a cytophagocytotic process in vivo as well as in vitro. In comparing Figures 10 and 11, one can easily notice that the sizes of the cross-sections of dendrites vary. There seem to be some variations in the mode of cytophagocytosis of the tip of dendrites. There were huge aggregates of melanosomes phagocytized in the keratinocyte of newborn animal, whereas small aggregates of

melanosomes were in the cell of adult animal (Figs. 10, 11). This can be explained as follows. The melanocytes of newborn guinea-pig epidermis possess a few, thick and short dendrites; on the other hand, those from the adult possess several, slender and long ones [5]. Therefore, the structural characteristics of the melanocyte may be one of the determining factors of the mode of cytophagocytosis. In the case of the hair follicle where keratinocytes are highly organized and melanocytes are well matured, the tips of dendrites of the melanocyte would be phagocytized by the cortical cells as a continuous event. Melanosomes appear to separate off from the pouch singly or in complexes of several melanosomes, during which time they acquire their outer membranes. These new outer membranes of melanosomes are assumed to come from the lysosomal membranes of the recipient keratinocytes.

Recently, Toda et al [14] have pointed out that in epidermal cells there is a striking tendency for small melanosomes to be complexed and for large ones to be dispersed as singles. Such a final distribution pattern could be formed without the action of melanocytes [2]. Small melanosomes were taken up in groups and remained as single membrane-bound aggregates within the cytoplasm of the recipient epidermal cells, while large melanosomes were incorporated as singles into keratinocytes and were dispersed singly within their cytoplasm [2,3,15,16].

From the experimental results obtained, it should be emphasized that the dispersion of melanosomes at the final stage, by which the final distribution pattern of melanosomes in the keratinocytes is determined, could only occur after the digestion of cytoplasmic constituents of melanocyte dendrites was completed and the final distribution pattern would be determined by the size of the individual melanosome.

From these light and electron microscopic observations, the transfer mechanisms of melanosomes from melanocyte to keratinocyte in vitro were assumed to be as follows (Fig. 12).

At the first stage, the melanocyte extends its dendritic process towards surrounding keratinocyte. Responding to the approach of dendritic process, the ruffling of the keratinocytic plasmamembrane becomes vigorous, the tip of the dendritic process penetrates into the keratinocyte, and enfolds with the cell membrane and villus-like cytoplasmic projections of the keratinocyte. In the second stage, the tip of the dendritic process appears to be squeezed and finally pinched off by the keratinocyte to form a pouch filled with numerous melanosomes. Ultrastructurally, the difference between the tip of the enfolded dendritic process and the pouch formed immediately after the process is pinched off, cannot be distinguished. There are two membranes: the inner membrane is derived from the melanocyte, and the outer one from the keratinocyte surrounding a mass of melanosomes. Cytoplasmic constituents including the melanosomes of the dendrite of the melanocyte are apparently intact. At the third stage, the pouch gradually moves towards the nucleus of the keratinocyte, during which time digestion takes place in this pouch. The inner membrane derived from the melanocyte and the other cytoplasmic constituents of the melanocyte, including the outer membranes of melanosomes, disintegrate completely. At the end of this stage, the pouch, which is located near the nucleus, consists of a single envelope membrane and numerous aggregated melanosomes without their outer membranes. At the fourth stage, these aggregated melanosomes seem to be released into the cytoplasm in the following ways. Primarily, individual melanosomes and groups of melanosomes scatter from the aggregate. The large aggregates of melanosomes may be separated first into several smaller clusters of melanosomes by way of developing invaginations of the single envelope membrane of the aggregate, after which melanosomes spill out from these small clusters, depending on their sizes. Individual melanosomes appear to again acquire outer membranes at the final stage.

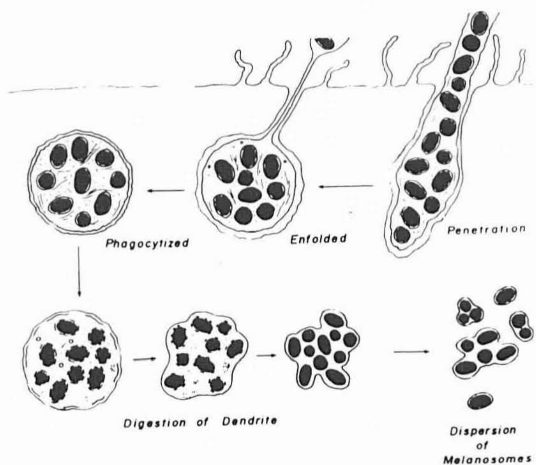


FIG. 12. Schematic illustration of melanosome transfer and dispersion.

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