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STUDIES ON GUINEA PIG SKIN CELL CULTURES[1]

V. *Co-culture of Pigmented Melanocytes and Albino Keratinocytes, a Model for the Study of Pigment Transfer*

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Abstract. Mixed cultures of melanocytes (M) and keratinocytes (K) are easily obtained from pigmented guinea pig ear skin. They are suitable for the study of pigment transfer from M to K. However, quantitation is difficult because many K are already loaded with pigment prior to cultivation. A technique is presented in which pigment-producing M are co-cultured with K of albino origin. Pigmented guinea pig ear skin is split with trypsin and basal cells including M are harvested. The cell suspension is treated with sodium citrate which prevents the attachment of K (but not of M) to the culture substrate. Ninety per cent pure M cultures are obtained. Five to seven days later, another basal cell suspension is prepared, this time from albino ear skin. This second suspension is seeded on top of the pigment-forming culture of M. The number of contacts between albino K and pigment-forming M increases as a direct function of time. Contrarily, the number of K which become pigmented increases until the fifth day of co-culture and decreases thereafter.

Key words: Melanocyte; Culture; Pigment; Pigmentation; Guinea pig

Pigment donation, i.e. the transfer of pigment from melanocytes to keratinocytes, is a major event in the mechanism of pigmentation (5, 16). Basically, this transfer is achieved through the phagocytosis of the tips of melanocytic dendrites by keratinocytes. Such 'cytophagocytosis' (13) has been documented both in vivo (4, 7, 11, 12) and, in vitro (2, 3, 8, 13, 14, 21).

However, quantitative approaches in vitro have been hampered by the fact that, in culture, most of the keratinocytes are already loaded with pigment at the time they are set in culture because they have benefited from pigment donation in vivo. Indeed, to accurately evaluate pigment transfer in vitro, the ideal situation would be that keratinocytes be devoid of melanin from the very inception of culture.

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In the present study we have developed a two-stage technique for the co-culture of melanocytes and keratinocytes. First, we isolated highly purified populations of melanocytes from pigmented guinea pig skin and second, we co-cultured these pigmented melanocytes with keratinocytes from albino animals.

MATERIALS AND METHODS

Animals

Ear skin from commercial tricolor and so-called albino (Hartley Strain) guinea pig (Evic Ceba, Blanquefort, France) was used. The day before cultivation, ears were depilated with a depilating wax, washed 3 times with 0.9% NaCl and once with 70% ethanol, and then treated with a mycostatic agent. On the following day, the animals were sacrificed. The ears were removed, washed as previously with 0.9% NaCl and 70% ethanol, and dried.

Culture of epidermal cells

The technique is derived from that previously described (17).

Separation of epidermis from dermis. Ears were stuck ventral side down on a sterile support with the aid of steristrips (3M Minnesota). Skin flaps from the dorsal aspect were dermatomized with an electrokeratome (Castroviejo) set at level 1. The skin flaps were then incubated in 0.15% trypsin in calcium and magnesium-free phosphate buffer (PBS) at 37°C.

Collection of melanocytes and keratinocytes. After 90 to 120 min of incubation with trypsin, the skin flaps were transferred to 3 ml per flap of culture medium consisting of Eagle's BME (Flow Laboratories) supplemented with 10% calf serum. The dermis was then lifted up with fine forceps. The cells of the basal layer which had remained attached to the dermis were extracted by shaking for 30 sec with an agitator (Vortex). The resulting cell suspension was then filtered through two layers of gauze and centrifuged for 10 min at 500 *g*. The supernate was discarded and the cells were suspended and washed with PBS. After washing, the cell pellet was treated with sodium citrate to separate melanocytes from keratinocytes.

Treatment of epidermal cells with sodium citrate. The pellet of cells harvested from pigmented skin as described above,

was suspended in 0.9% NaCl solution containing 0.8% sodium citrate for 3 min. Usually, 1.5 ml of sodium citrate solution was used to suspend the pellet of cells harvested from two skin flaps. Following this treatment, an equal amount of culture medium was added. Cells were counted with a hemocytometer and cell viability was estimated by trypan blue exclusion. The cell suspension was adjusted to 5×10^5 cells/ml. Such adjusted suspension contained about 12 500 melanocytes.

Co-culture of pigmented melanocytes and albino keratinocytes. Two ml per tube of an epidermal cell suspension containing 5×10^5 cells per ml prepared from pigmented ear skin of tricolor animals and treated with sodium citrate were seeded onto glass coverslips (12 × 32 mm) in Leighton tubes. Twenty-four hours later the medium was removed and fresh medium was added. The medium was thereafter changed every 2 to 3 days. After 7 days of culture, a second epidermal cell suspension was prepared from the ear skin of albino animals. Two ml per tube of this suspension containing 10^6 cells per ml, prepared as above but not treated with sodium citrate, were introduced into the tubes and the cells were allowed to sediment on the same coverslips on which the first cultures had been made a week before.

Light and electron microscope studies

Melanocyte and keratinocyte cultures were fixed for light microscope examination by dipping the coverslips in methanol/acetic acid (3:1) fixative, and stained with Giemsa.

For electron microscope studies, they were fixed in 3% glutaraldehyde in Sörensen phosphate buffer 0.1 M at pH 7.2 for 10 min, washed with the phosphate buffer and post-fixed with 1% osmium tetroxide in the same buffer for 10 min. Dehydration and embedding were carried out on cells still attached to the coverslips, as previously described (10). The cell sections were stained with uranyl acetate and lead citrate.

Autoradiography

Cell cultures were labelled with 3H-thymidine at the final concentration of 10 μCi/ml (Specific activity 10 Ci/mM, C.E.A., Saclay, France) in the culture medium for 3 hours at 37°C. After fixation with 3% glutaraldehyde, the cells were treated with 2% perchloric acid for 20 min at 4°C, and dipped in Ilford L4 emulsion. After 2 weeks of exposure, the autoradiographs were developed with D19 B Kodak developer.

RESULTS

I. *Purified Melanocyte Cultures*

A. *Morphology of the cultures*

1. *Control cultures not treated with sodium citrate.* Epidermal cells sedimented down onto the glass support. After 24 hours a number of cells were firmly attached, though a substantial part of the cell population could be removed by gentle washing. Thus, by changing the culture medium at this time a large number of cells were discarded. It has been our experience that those cells which had not attached by 24 hours would not do so in the following hours and that to keep them longer might be deleterious to the culture.

After 3–4 days, numerous small sheets of epithelial-like cells were scattered over the coverslip. Many branched cells identifiable as melanocytes because of their shape and pigment content were visible. They frequently formed bridges between adjacent groups of growing cells. Many contacts between melanocytes and keratinocytes were found. Most keratinocytes contained pigment.

2. *Cultures treated with sodium citrate.* Epidermal cells sedimented onto the glass support, as above. After 24 hours, the number of cells which were firmly attached and which resisted the change of culture medium was far less than in untreated cultures. This diminution in number was due to the loss of keratinocytes. After 3–4 days, isolated melanocytes were visible throughout the coverslip. They appeared like massive, pigmented elements. There were no epithelial-like cell sheets. Only rare isolated keratinocytes could be seen (Fig. 1 *a*).

At 5 days, the growth of these keratinocytes was very limited. Melanocytes developed club-shaped cytoplasmic processes which, on many occasions, extended from one cell to the other, giving a network appearance to the culture (Fig. 1 *b*, *c*, *d*). Under the electron microscope, these melanocytes exhibited typical melanosome structures in their cytoplasm. These melanosomes could be seen at all stages of maturation, including melanosome stages 1, 2, 3 and fully melanized stage 4 (Fig. 2). The Golgi zone was well developed. Mitochondria were small and few in number. The nucleus generally contained two nucleoli and chromatin clumps located close to the inner nuclear membrane.

At 7 days, cultures composed of 90% or more melanocytes were routinely obtained. For the purpose of co-culture, which will be described below, melanocyte cultures which had not reached this degree of purity were discarded.

B. *Parameters involved in the purification of melanocyte cultures*

1. *Effect of trypsin.* The number of melanocytes present on the coverslip after 5 days of culture varied with the duration of trypsinization. This number culminated around 700 for trypsinization times of the order of 110 ± 10 min. Trypsinization longer than 2 hours resulted in a sharp decrease in melanocyte number.

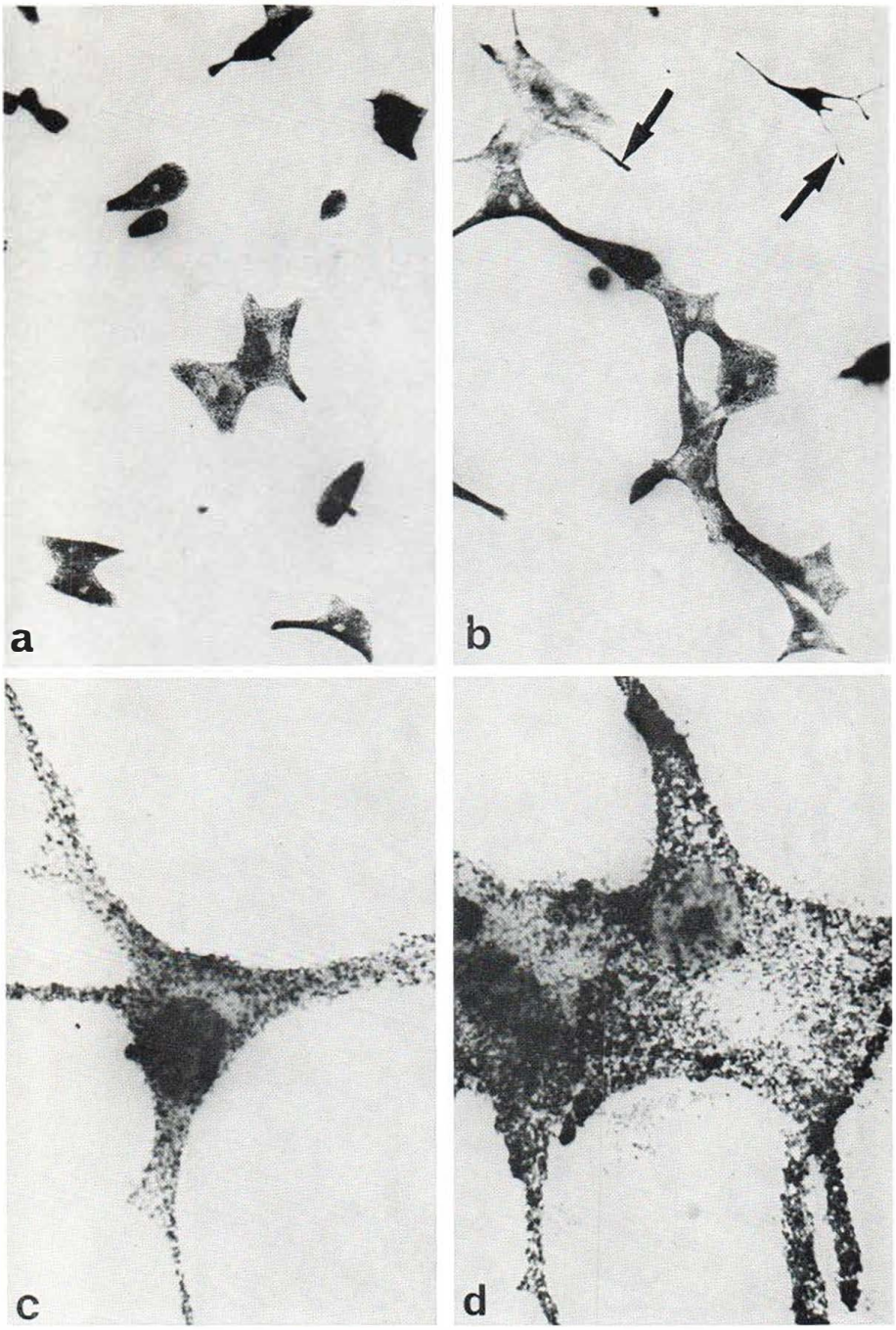

Fig. 1. Purified coverslip cultures of pigmented melanocytes. (*a*) Three days after the epidermal cell suspension was treated with sodium citrate and seeded, most of the cells which have remained attached to the coverslip are massive, pigmented melanocytes. ×300. (*b*) At 5 days, melanocytes tend to form irregular networks. Arrows point to melanocytic dendrites. ×300. (*c, d*) High magnifications of melanocytes after 7 days in culture. The nucleus is small. There is a large clear zone near the nucleus. A few melanin granules are lying free on the coverslip. This is due to fixation. ×1 330.

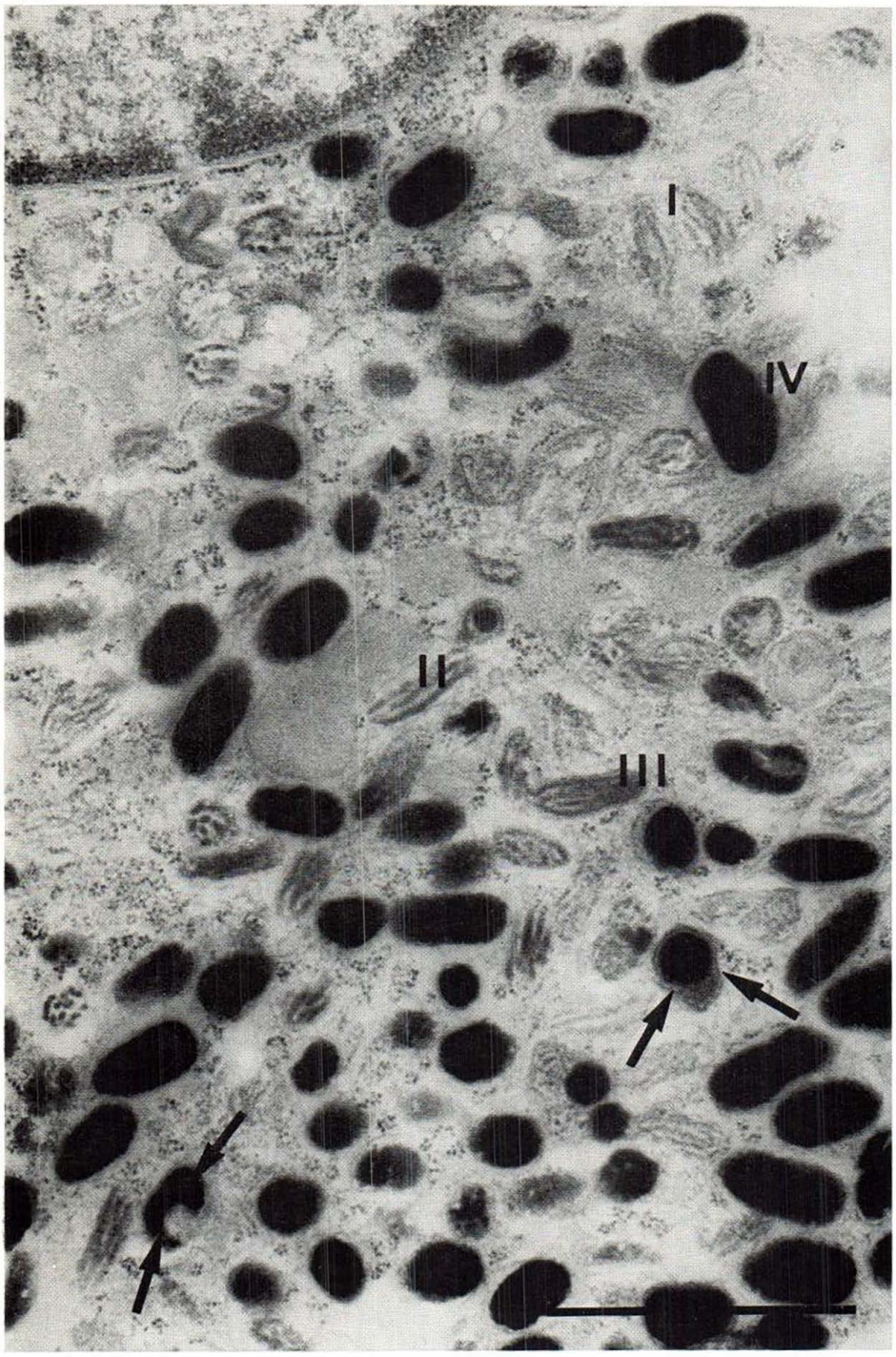

Fig. 2. Ultrastructure of melanocytes isolated by treatment of the epidermal cell suspension with sodium citrate. Electron micrograph of part of a melanocyte at 8 days of culture. Melanosomes at stages I, II, III and IV are seen. Arrows point to mature melanosomes, stage IV, which exhibit incomplete, irregular melanization. × 42 000.

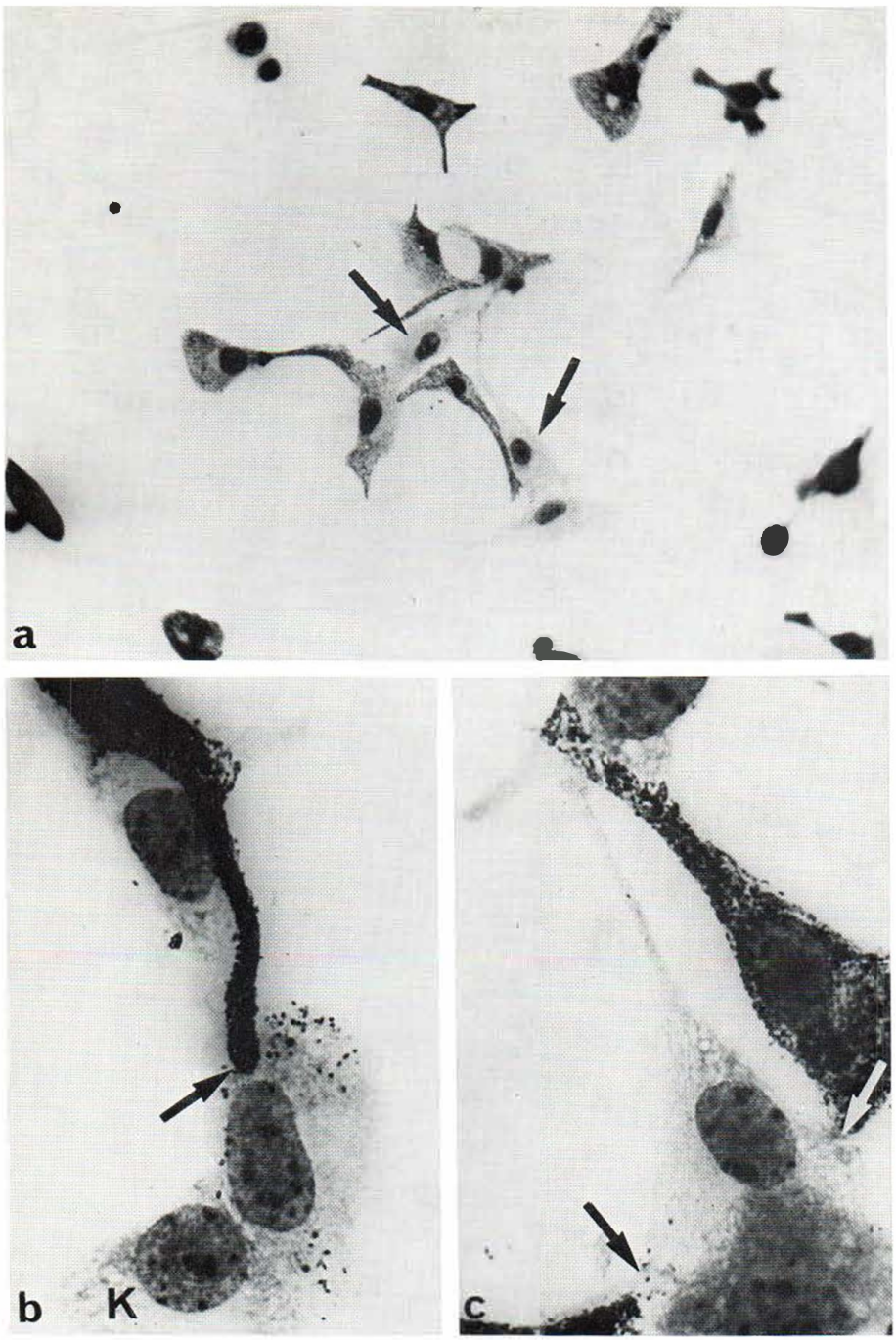

Fig. 3. Co-culture of pigmented melanocytes and albino keratinocytes. (*a*) After 7 days of culture, a suspension of albino epidermal cells was seeded on top of a purified melanocyte culture. Pigmented melanocytes and albino keratinocytes were then co-cultured for 2 days. Arrows point to albino keratinocytes. × 155. (*b*) This picture shows the contact of a melanocytic dendrite and a keratinocyte (*arrow*). This keratinocyte contains pigment granules. Another keratinocyte (*K*) is not in contact with the tip of the dendrite. It is devoid of pigment granules. × 1 000. (*c*) Here, a keratinocyte is in contact with two melanocytic expansions. The black arrow points to a contact associated with pigment transfer; the white arrow points to a contact which is not associated with pigment transfer. × 1 000.

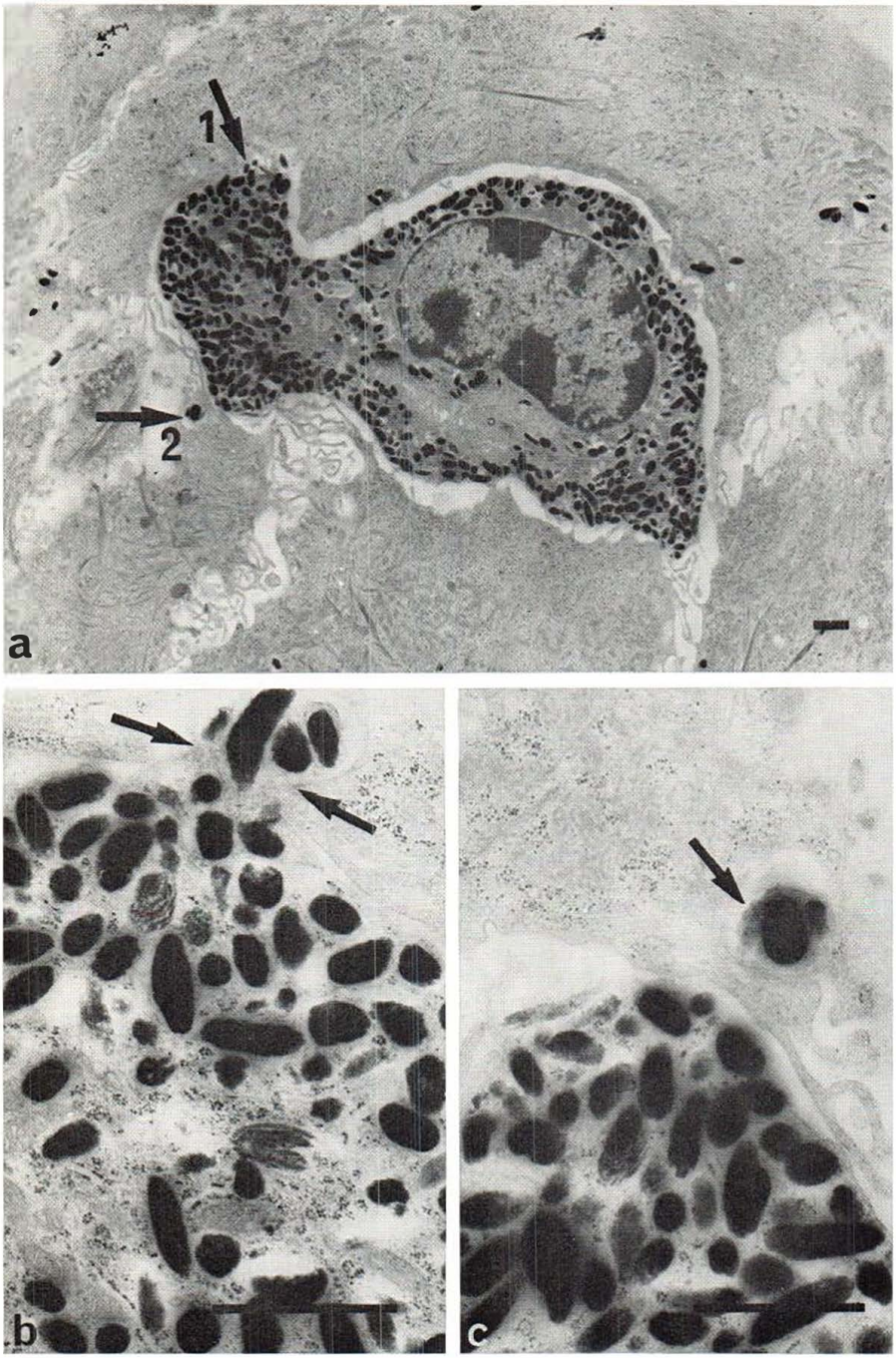

Fig. 4. Electron microscopic views suggesting cytophagocytosis. (*a*) A melanocyte is surrounded by several keratinocytes. A clear space can be seen around the melanocyte. In some places, microvilli extend from the surface of the keratinocytes through this clear space. Arrows point to groups of melanosomes which seem to be in the process of being transferred to the keratinocytes. ×4 250. (*b*) Magnification of a lower section of the zone indicated on Fig. 4*a* by arrow number 1. Arrows point to pinching off of a group of melanosomes imbedded in melanocyte cytoplasm. ×22 000. (*c*) Magnification of the zone indicated on Fig. 4*a* by arrow number 2. A group of melanosomes imbedded in melanocyte cytoplasm are enwrapped by cytoplasmic expansions originating from the surface of the adjacent keratinocytes. ×22 000.

2. *Effects of sodium citrate.* The effect of sodium citrate concentration in 0.9% NaCl solution was sharply delimited: below 0.6% it had no effect and above 1% the melanocyte viability was affected. The best concentration was 0.8%. The number of melanocytes was highest after 5 days of culture for a treatment duration of 3 min. Six min of contact of the epidermal cell suspension with 0.8% sodium citrate had a deleterious effect upon the viability of melanocytes, as estimated by cell morphology (lack of cell flattening and disruption of cytoplasmic boundaries).

3. *Other parameters.* (*a*) Preliminary data showed that epidermal cells were sensitive to EDTA. Treatment of epidermal cell suspensions with 0.02% EDTA in place of sodium citrate was enough to prevent the attachment of keratinocytes. However, in such condition melanocytes did not adhere to the glass either.

(*b*) On the contrary, treatment of epidermal cell suspensions with 0.04 EGTA had no appreciable effect on the selection of any of the two cell types.

(*c*) Mixtures of sodium citrate and EGTA or EDTA did not yield better selection of melanocytes than that obtained with sodium citrate alone.

(*d*) Variation of serum concentration in the culture medium was found to have little effect on the attachment of melanocytes. In particular, when a serum-free medium was used during the first 18–20 hours of culture, large numbers of melanocytes could be seen, firmly attached to the coverslip. By contrast, the attachment of keratinocytes was highly dependent upon serum concentration. Below 5%, the number of keratinocytes which attached to the glass support was progressively reduced. In serum-free medium, very few keratinocytes adhered to the coverslip.

II. *Co-cultures of Pigmented Melanocytes and Albino Keratinocytes*

A. *Morphology of cultures*

When a suspension of albino epidermal cells was added to a culture of pigmented melanocytes, numerous contacts between the two cell types readily occurred (Fig. 3*a*). One single melanocyte was frequently seen in contact with two or more keratinocytes.

Under the light microscope, two types of contacts were seen: one which was associated with the presence of pigment granules in the keratinocyte (Fig. 3*b*), and another which was not (Fig. 3*c*). Under the electron microscope, a clear space could be seen between melanocytes and surrounding keratinocytes. Microvilli extended from keratinocytes through this clear space (Fig. 4a). Photographs suggesting the pinching-off of bits of melanosome containing cytoplasm by these microvilli have been taken (Fig. 4*b*, *c*).

Quantitatively, three parameters were considered according to time:

(*a*) The total number of contacts between melanocytes and keratinocytes. This number was estimated in scoring all clearly identifiable contacts, irrespective of the presence or absence of pigment within the keratinocyte.

(*b*) The number of melanocytes present on the coverslip.

(*c*) The number of contacts in which melanin granules could be detected in keratinocytes. This number represented the total number of cell contacts which resulted in pigment transfer.

A "contact index" was given by the ratio of *a* to *b*, i.e., total number of contacts between melanocytes and keratinocytes/number of melanocytes on the coverslip × 100. Similarly, a "transfer index" was given by the ratio of *c* to *a*, i.e., number of contacts with pigment transfer in keratinocytes/total number of contacts × 100. The variations of both contact and transfer indices were plotted against time (Figs. 5 and 6). The "contact index" increased from day 3 to day 9 after the onset of the co-culture; by contrast, the "transfer index" increased until the 5th day and then decreased significantly.

B. *Growth capacity of melanocytes and keratinocytes in co-culture*

Autoradiographic studies on 3-day-old co-cultures labelled with ^{3}H-thymidine, revealed that around 30% keratinocytes were labelled. No labelling was detected in melanocytes.

DISCUSSION

Partially purified cultures of non-malignant melanocytes have been obtained in various laboratories (8, 18). However, no specific technique is available for the consistent isolation of purified populations of such cells. The use made in the present study of sodium citrate as selecting agent stems from the work of Billingham & Reynolds in 1952 (1), in which they used 0.8% sodium citrate in saline to disperse epidermal cells in order to prepare epidermal cell suspensions for transplantation.

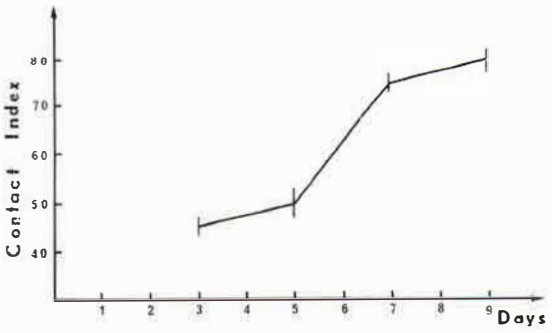

Fig. 5. Variation of the contact index according to time. This index increases from day 3 to day 9 after the start of co-culture.

Back in 1965, one of us noticed that melanocytes attached to a glass support more rapidly than did keratinocytes. Indeed, partial selection of melanocytes could be achieved in Cruickshank's culture chambers by letting the epidermal cell suspension first sediment on the glass side of the chamber for 15 to 18 hours and then by inverting the chamber to allow those cells which had not attached to the glass to adhere to the opposite, plastic surface of the chamber (14). Since the attachment of cells to glass is largely dependent upon the presence of divalent cations like Ca^{++} and Mg^{++} in cell environment (9, 19), it was thought that the difference in attachment ability might be related to different susceptibilities of these cells to the presence of Ca^{++} and/or Mg^{++}. Therefore, if these cations were displaced at the proper time, it could be that keratinocytes would become completely loosened, whereas melanocytes would remain able to adhere to the glass support. The experiments reported in this paper show that the use of sodium citrate reduces the number of keratinocytes which attach to the glass to such a point as to permit selection of a highly purified population of melanocytes. Coverslip cultures composed of 90% or more melanocytes were easily obtained on a routine basis. However, the number of selected melanocytes is very low. Not more than 600 to 700 melanocytes were selected out of a cell suspension containing 10^6 elements. Since there are about 12 500 melanocytes in an epidermal suspension of 5×10^5 cells, this means that no more than 1 out of 40 melanocytes or so will actually adhere to the glass and flatten out. This indicates first, that the population of melanocytes is heterogeneous and second, that in vitro cultures prepared according to the technique described herein are only partially representative of the in vivo situation.

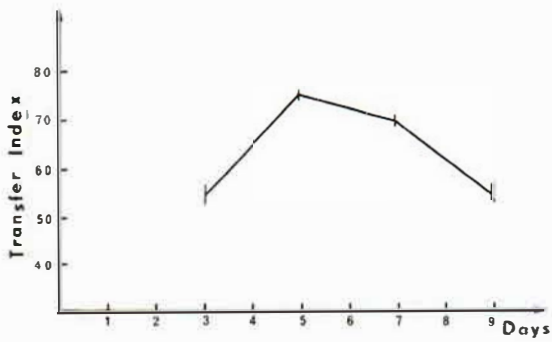

Fig. 6. Variation of the transfer index according to time. The index increases up to 5 days after the start of the co-culture and decreases thereafter.

It is of interest to note that the values of the "transfer index" as defined in this study are different from those of the "transfer index" of Klaus (8). In Klaus' calculation, the maximum transfer activity was reached after 13 to 14 days of culture, and culminated at 24.8. In the experiments reported herein, transfer activity peaked at the 5th day of co-culture and reached 70%. There is little difference between these two studies as regards the time at which maximum transfer activity occurred. Since the albino cell suspension was added to the melanocyte culture after 7 days of cultivation and the peak of transfer activity occurred after 5 days of co-culture, the total time spent by melanocytes in vitro amounts in fact to 12 days in our experiments. As far as the transfer activity itself is concerned, however, our values are much higher than those of Klaus. One may wonder whether this reflects technical disparity. In our study, we counted the number of contacts associated with the presence of pigment in keratinocytes as positive and these not associated, as negative. In so doing, we estimated the result of the transfer process. In Klaus' experiments, the parameters chosen to evaluate the transfer activity were not the result of but rather the active part of the process, namely, the withdrawal of dendrites and melanin package digestion. This latter procedure is perhaps more difficult since it requires some effort in interpretation, whereas in our system we deal with a yes/no situation. Basically, however, there is no fundamental difference between the two modes of evaluation.

On the other hand, the definition of the melanocyte–keratinocyte pair in Klaus' paper takes into account the fact that one single melanocyte can be

associated with more than one keratinocyte. In like manner, we have counted all contacts whatever the number of keratinocytes involved. Thus, technical differences do not seem to explain the much higher activity observed in our system.

One possibility is that the presence of melanin granules in keratinocytes depresses the phagocytic ability of this cell. It would then be understandable why the transfer of pigment was higher in our experiments (in which phagocytosing cells were pigment free) than in Klaus' experiments in which cultures were derived from pigmented skin where many if not all keratinocytes already contained some pigment at the time of cultivation.

Finally, this study furnished an opportunity to examine contacts between melanocytes and keratinocytes in vitro, by means of the electron microscope. In addition to confirming previous findings (6, 15, 20), attention is attracted to microvilli, which extend from the surface of keratinocytes and seem to play a part in the cytophagocytosis process by which melanosomes are transferred from the melanocyte to the keratinocyte.

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REFERENCES

1. Billingham, R. E. & Reynolds, J.: Transplantation studies on sheets of pure epidermal epithelium and of epidermal cell suspensions. Br J Plast Surg *23:* 25, 1952.
2. Cohen, J. & Szabo, G.: Study of pigment donation in vitro. Exp Cell Res *50:* 418, 1968.
3. Cruickshank, C. N. & Harcourt, S. A.: Pigment donation in vitro. J Invest Dermatol *42:* 183, 1964.
4. Drochmans, P.: Etude au microscope électronique du mécanisme de la pigmentation mélanique. Arch Belg Dermatol *16:* 155, 1960.
5. Fitzpatrick, T. B., Miyamoto, M. & Ishikawa, K.: The evolution of concepts of melanin biology. Arch Dermatol *96:* 305, 1967.
6. Gazzolo, L. & Prunieras, M.: Melanin granules in keratinocytes in vitro. J Invest Dermatol *51:* 186, 1968.
7. Hori, Y., Toda, K., Pathak, M. A., Clarck, N. H. & Fitzpatrick, T. B.: A fine-structure study of the human epidermal melanosome complex and its acid phosphatase activity. J Ultrastruct Res *25:* 109, 1968.
8. Klaus, S. N.: Pigment transfer in mammalian epidermis. Arch Dermatol *100:* 756, 1969.
9. Morasca, L., Balconi, G., Dolfini, E. & Oldani, C.: Effect of trypsin and ethylene diamine tetra acetic acid (EDTA) on the rate of adhesion of K B cells. Experientia *29:* 909, 1973.
10. Moreno, G. & Vinzens, F.: Effet de la microirradiation ultraviolette sur différentes parties de la cellule. I. Etude en microscopie électronique sur coupes en série transversale et sagittale. Exp Cell Res *56:* 75, 1969.
11. Mottaz, J. H. & Zelickson, A. S.: Melanin transfer: a possible phagocytic process. J Invest Dermatol *49:* 605, 1967.
12. Parakal, P. F.: The transfer of premelanosomes into the keratinizing cells of albino hair follicles. J Cell Biol *35:* 473, 1967.
13. Prunieras, M., Leung, T. K. & Colson, P.: Dissociation et recombinaison de l'épiderme de Cobaye adulte. Ann Derm Syph *91:* 23, 1964.
14. Prunicras, M.: La Culture de l'Epiderme. I Vol. SPEI publ., Paris 1965.
15. — Interactions between keratinocytes and dendritic cells. J Invest Dermatol *52:* 1, 1969.
16. — Physiologie du mélanocyte de la peau de Mammifère. Path Biol *19:* 531, 1971.
17. Regnier, M., Delescluse, C. & Prunieras, M.: Studies on guinea pig skin cell cultures. I. Separate cultures of keratinocytes and dermal fibroblasts. Acta Dermatovener (Stockholm) *53:* 241, 1973.
18. Riley, P. A.: Melanin and melanocytes, *in* The Physiology and Physiopathology of the Skin (ed., A. Jarrett) Vol. 3, p. 1101–1130. Academic Press, London, 1974.
19. Waymouth, C.: To disaggregate or not to disaggregate. Injury and cell disaggregation, transient or permanent? In Vitro *10:* 97, 1974.
20. Wikswo, M. A. & Szabo, G.: Studies on the interactions between melanocytes and keratinocytes with special reference to the role of microfilaments. Pigment cell, series editor: V. Riley, Vol. 1, pp. 27–38; Mechanisms in Pigmentation, volume editors V. J. McGovern & P. Russell. S. Karger, publ., Sydney, 1973.
21. Wolff, K., Jimbow, K. & Fitzpatrick, T. B.: Experimental pigment donation in vitro. J Ultrastruct Res *47:* 400, 1974.

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ADDENDUM

Since this paper was sent for publication, a study by P. A. Riley appeared in the Br. J. Dermat *92*; 291, 1975, which deals with the same subject. According to Riley, the culture of epidermal cells in the presence of high tension oxygen would permit to select purified populations of melanocytes which would incorporate thymidine and enter mitosis in vitro. Since, in our procedure, purified melanocytes do not multiply, it would be an indication that oxygen tension might be a key factor in the induction of growth of melanocytes in culture.