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The culturing of gerbil epidermal cells is described for the first time. Cells grew first as clumps, formed monolayers of proliferating basal cells and then differentiated, giving rise to a stratified epithelium. To regulate the process of proliferation and differentiation, two kinds of medium were used: the standard one and a low-Ca^{++} medium. Under these two conditions, cultured gerbil cells were compared with mouse epidermal cell cultures. The cultures maintained in the low-Ca^{++} medium showed greater differences between the two species, the gerbil epidermal cells displaying a greater proliferative capacity and maintained capacity to differentiate, than did the murine cells. It is believed that this *in vitro* model may lead to a better understanding of the difference between the two species *in vivo*, and might explain their differing susceptibility to cutaneous tumour induction. *Key words: Epidermis; Keratinocyte; Growth and differentiation*.

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The skin has proved quite useful as a research material with which to increase our knowledge of a number of biological processes. Its capacity for proliferation and differentiation, its relatively ample availability and the possibilities of manipulation under various conditions, all make epidermis an excellent model by which to study the effect of a number of factors controlling those two functions.

The possibility of *in vitro* cultivation of skin cells, such as keratinocytes, led to a wealth of relevant information allowing not only a better understanding of the normal and abnormal development of tissue, the action of chemical carcinogens, and the proliferative process, but also to a better comprehension of the regenerative process.

There is a general consensus that either as an auxiliary method or as a primary investigative tool, epidermal cell cultures can be used in nearly all areas of cutaneous research (1). Epidermal cell cultures of a number of species have already been established, such as Man (2, 3), mouse (4–7), rat (8, 9), guinea pig (10, 11) and rabbit (12). The objective of the present work was to establish a primary culture model of gerbil epidermal cells, describing its behaviour, comparing it with the process of growth and differentiation already described for other species and, in this particular work, to compare the characteristics of gerbil with murine epidermal cultures set up in parallel.

The importance of establishing gerbil epidermal cultures derives from the difference in the propensity of gerbil skin to develop experimental tumours, in comparison with mouse skin (13). It was thought that gerbil skin cells might possess some intrinsic characteristic that would explain its resistance to the induction of chemical carcinogenesis.

MATERIAL AND METHODS

Animals

BlOA mice, of either sex were used throughout this study. Gerbils (*Meriones unguiculatus*), of either sex, were originally obtained from the animal house of the Pathology Department at the Fluminense Federal University, though the animals used in the present study were also bred in our own animal house.

Cell culture

Epidermal cells, obtained from the skin of 1–3 day-old pups, were washed with povidine for 15 min, followed by 70% ethanol. Whole skin was removed under sterile conditions and processed using a modification of the method described by Yuspa et al. (7). After incubation in trypsin, the skin was transferred to fresh medium with serum and small fragments were excised without separating dermis from epidermis. These fragments were stirred in complete medium for 30 min at 37°C to dissociate the cells which were then processed as in the original method. Two different cell culture media were used: M199 (Sigma Chemical Corp.) with 10% Fetal Calf Serum (FCS) (Hyclone) and F12 (Sigma) containing only 0.04 mM Ca^{++}, also with 10% FCS. Both media were supplemented with penicillin, streptomycin and fungizone. Cell suspensions were prepared in 199 medium, 5 ml medium for each animal skin, and plated into Nuclon plastic Petri dishes (Nunc) 3 cm in diameter, in a volume of 2.5 ml per plate. The cultures were kept at 33°C or 37°C in a humidified atmosphere of 5% CO_2. After 24 h the medium was renewed and some cultures received M199 or fresh F12 medium and from this day onward the medium was changed three times a week.

Cell proliferation

Proliferation was assessed by [^{3}H] thymidine incorporation into the DNA. Cell suspensions in M199 were plated into a 96-well plate (Nunc) in a volume of 200 µl per well. After the first 24 h the non-attached cells were removed and fresh medium (M199 or F12) was added to the cultures. On days 2, 4, 6, 8, 10, 16 and 21, [^{3}H] thymidine (0.5 µCi) was added to each well and left for a 6 h pulse, after which the cultures were trypsinized (20 µl) of 25% trypsin in saline) and harvested for radioactivity counting.

Differential trypsinization

The technique used was that described by Milo et al. (14). Briefly this was as follows: on day 5 following seeding, the fibroblasts were selectively detached by adding to the cultures 0.25% trypsin in saline.

Fibroblast detachment was observed at room temperature using phase microscopy. Ideally, trypsin treatment lasted about 3 min.

Cell staining

The cells were stained with H.E., Sudan III and PAS. To identify cornification, epithelial populations from the epidermis were stained with rhodanile blue, as described by Rheinwald & Green (15). The cells were fixed in 10% buffered formate and a 1% preparation of

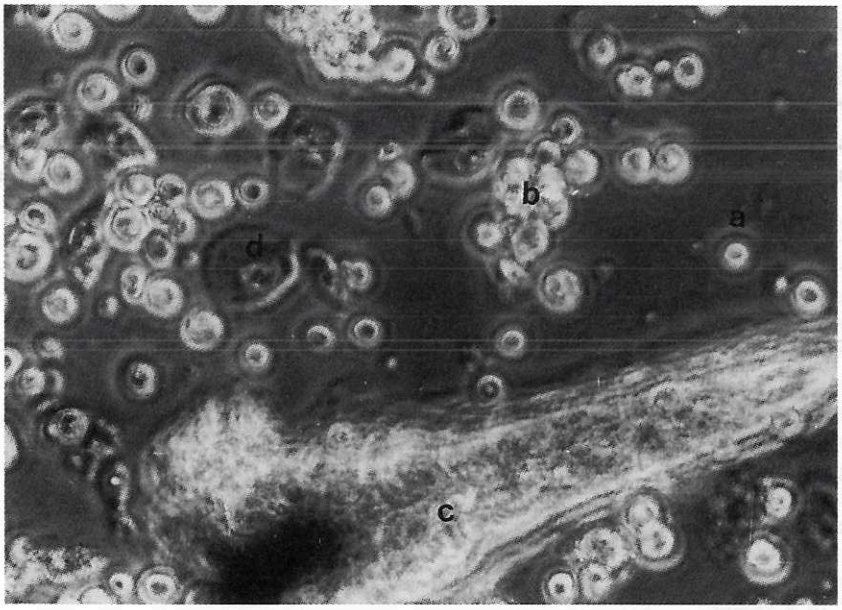


Fig. 1. Suspension of gerbil epidermal cells after one hour in culture in standard medium. Isolated cells (A), cell clumps (B), pilous follicle (C) and mature cells (D) (×1200).

rhodanile blue was added to the culture. After 30 min, the cells were rinsed with several washes of water until the pink colour could no longer be seen. The cultures were then examined by light microscopy.

RESULTS

Isolation of cells

In preliminary experiments we attempted to follow the isolation method described by Yuspa et al. (7), using gerbil skin. However, cell recovery from the basal layer was extremely poor, even though the cell suspension obtained was unicellular. From then on, a modification of the method was used. This technique afforded a good yield of basal cells having proliferative capacity (Fig. 1), despite the presence of fibroblasts, melanocytes and mature cells from the upper layers. However, this contamination did not affect the cultures, as can be observed in the following results.

Epidermal cell cultures

To study the effects of Ca^{++} on the growth of gerbil epidermal cells, two types of medium were used: standard culture medium (0.185 g/l Ca^{++}) and low calcium medium (0.044 g/l).

Cultures in standard medium

Initially, cells tended to aggregate and to form islands (Fig. 2). These islands consisted of small polygonal cells having large nuclei, prominent nucleoli and striking perinuclear granulation, clear nuclear definition and distinct outer cell boundary lines. Their nucleoli varied in number, size and form. The cells had a high nuclear/cytoplasmic ratio. They were disposed in a compacted layer under larger cell clumps apparently formed by living cells, as viewed by phase-contrast microscopy.

Cells spread during the first day in culture, and confluent

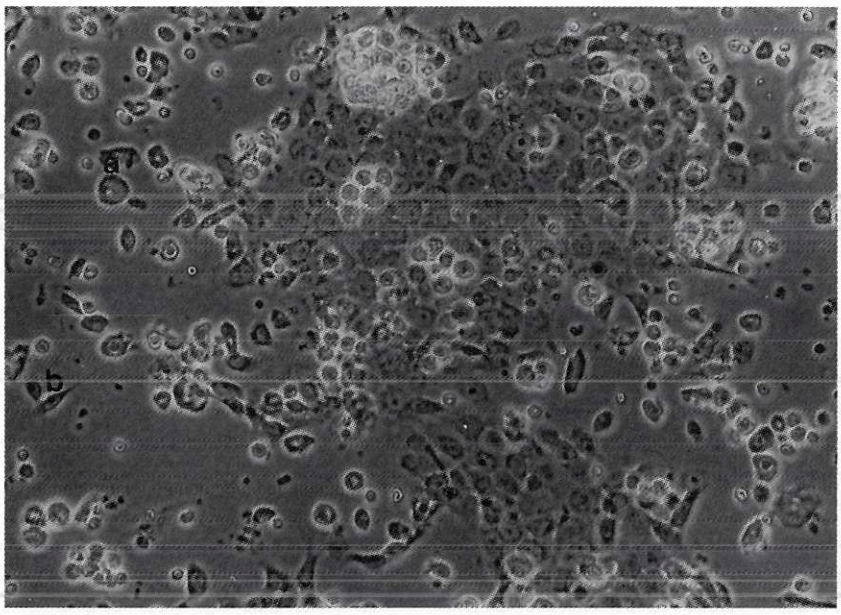


Fig. 2. Gerbil epidermal cells after 24 h in culture in standard medium. Mature cells (A), fibroblast (B) and epithelial cell islands (×480).

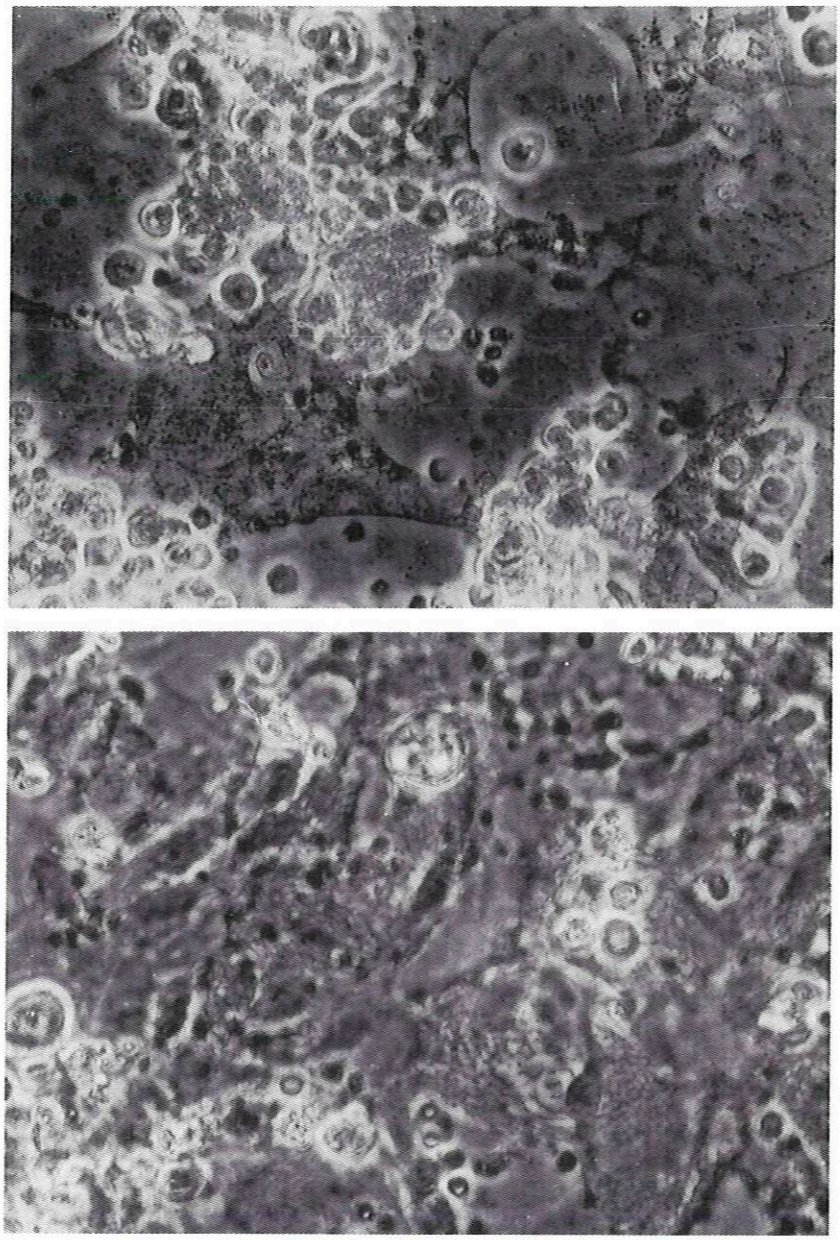


Fig. 3. Gerbil and mouse epidermal cells after 5 days in culture in standard medium. (A) Gerbil cultures; presence of a second monolayer constituted of epithelial cells with dense granulation. (B) Mouse cells; formation of filamentous nets in cytoplasms (×1200).

monolayers of polygonal epidermal cells could be seen after 3–4 days.

From the 5th day onwards, cultures behaved differently, depending on the origin of the cells and the culture medium employed. When medium 199 was used, few differences were observed between gerbil skin and mouse skin in cell cultures. The most noticeable characteristic of gerbil epidermal cells cultures was the development of a second monolayer consisting of large epithelial cells with dense granulation upon the original monolayer (Fig. 3A), whereas mouse cultures no longer displayed this granular appearance, and were already exhibiting filamentous nets (Fig. 3B).

The stratification process proceeded with gerbil epidermal cells exhibiting filamentous nets and distinct desmosomes in large quantities in the cell cytoplasms on the 8th day in culture (Fig. 4).

At a more advanced stage, after 11–13 days, the central region of the culture displayed pronounced stratification, but well demarcated regions persisted at the periphery, consisting of large polygonal cells with a clearly defined cellular boundary. No intercellular bridge could be seen between these cells. In the central region the cultures exhibited a high degree of terminal differentiation which ultimately led to the formation of a multicellular stratified structure. The striated pattern of the cells developed further, giving the whole cell sheet a "crackled" appearance. These fibrillar structures did not cover the whole cell but tended to leave the nuclear area relatively free and also formed charateristic tonofibrillar connections at intercellular boundaries. These were seen in both gerbil and mouse epidermal cultures and may reflect the appearance of the prickle cell layer of the epidermis (Fig. 5). From then on, no differences between the cultures of the two species could be observed.

After 25–30 days in culture, the stratification process pro-

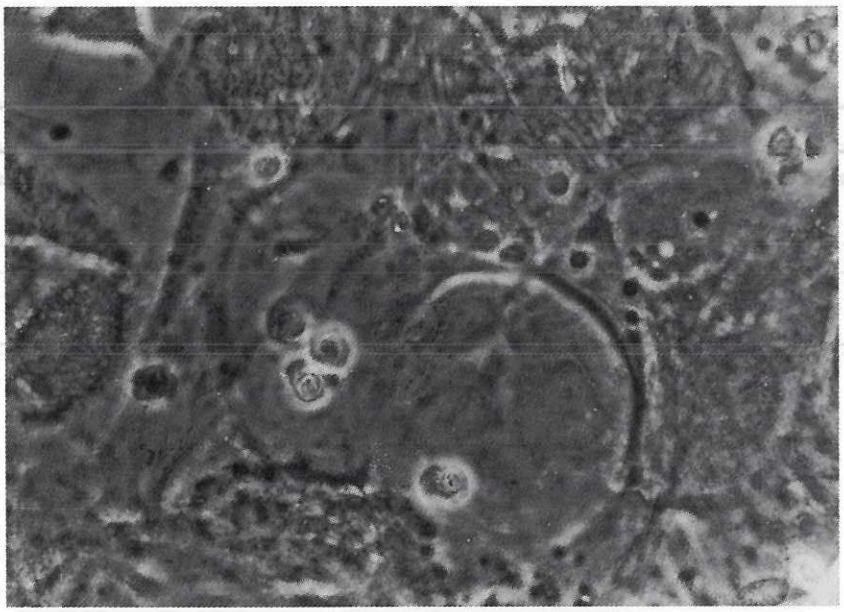


Fig. 4. Gerbil epidermal cells after 8 days in culture in standard medium. Formation of fibrillar structures in cell cytoplasms (×1200).

ceeded with the development of a third layer of released cells that conferred a three-dimensional appearance to the cultures (Fig. 6).

The cells that formed these upper layers had a different morphology. They had thickened membranes, their nucleoli were missing or pyknotic, and they showed dense cytoplasmic granularity. At this stage, observation of basal cells by phase-contrast microscopy became more difficult due to the formation of these upper layers. Despite the fact that only indistinct contours of keratinocytes were visible, some dark melanocytes with their dentritic cell shape could be clearly seen.

With the progressive growth of these upper layers, the number of adherent cells in culture began to decrease until the cultures degenerated completely.

Cultures in low-Ca^{++} medium

It has been suggested that in cultures of other species, low-Ca^{++} medium shifted the proliferation-differentiation balance towards proliferation. This was studied in gerbil cultures by replacing the standard medium 24 h after plating, by a medium with a low Ca^{++} concentration (F12 medium).

Under these culture conditions, gerbil and mouse epidermal cells exhibited, from the fifth day onward, distinct morphologic characteristics.

In cultures using gerbil cells, basal cells attached to the bottom of culture dishes to form a monolayer reaching confluence. These cells had the same morphologic characteristics seen at the beginning of the culture with standard medium, but there were large intercellular spaces (Fig. 7).

At this stage, despite the fact that some mouse epidermal

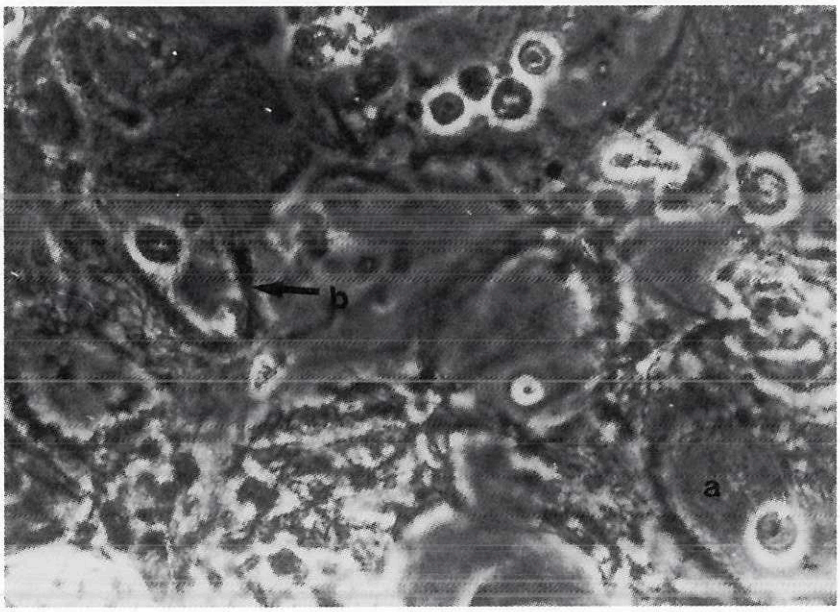


Fig. 5. Gerbil epidermal cells after 13 days in culture in standard medium: cells are larger, with more marked fibrillar structures (A); cells with thickened cytoplasmic membrane (B) (×1200).

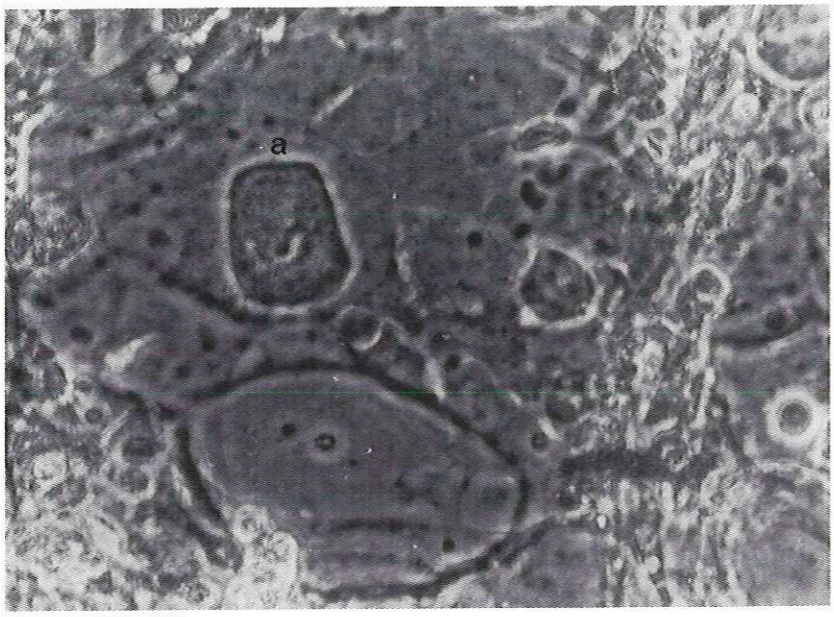


Fig. 6. Gerbil epidermal cells after 28 days in culture in standard medium; cells with thickened cytoplasmic membrane (A), three-dimensional appearance (×1200).

cells possessed the early characteristics of gerbil cells, most of the cells showed irregular forms, starting a stretching process that became more apparent as the culture proceeded (Fig. 8A).

After 9 days in culture, gerbil epidermal cells maintained the same morphologic characteristics seen at the beginning of the culture, but small stratified regions could be seen. In this phase, rare cells, somewhat larger than monolayer polygonal cells, occurred in gerbil cultures; these cells had a cytoplasm with numerous vacuoli and their nucleus was displaced to the periphery; these cells were not arranged in groups but were interspersed among the polygonal cells of the monolayer (Fig. 8B).

The cytoplasm of mouse epidermal cells became larger as culturing proceeded. Sometimes, two or more nuclei were seen together in the same cell, desmosomes were not visible, and large dense granules could be seen dispersed by the cultures. In this phase, the vacuolated cells observed in gerbil cultures were also present in mouse cultures (Fig. 9). The difference between the cultures from these two species was still quite distinct.

In gerbil cultures at about the 19th day there were still numerous small polygonal cells forming a monolayer, stratification areas were slightly larger and monolayer cells were larger and more granulated (Fig. 10). At the same time, mouse cells persisted with their spread form. This phase of mouse culture was characterized by large and dense granules agglomerated in areas with vacuolated cells, these granules first being

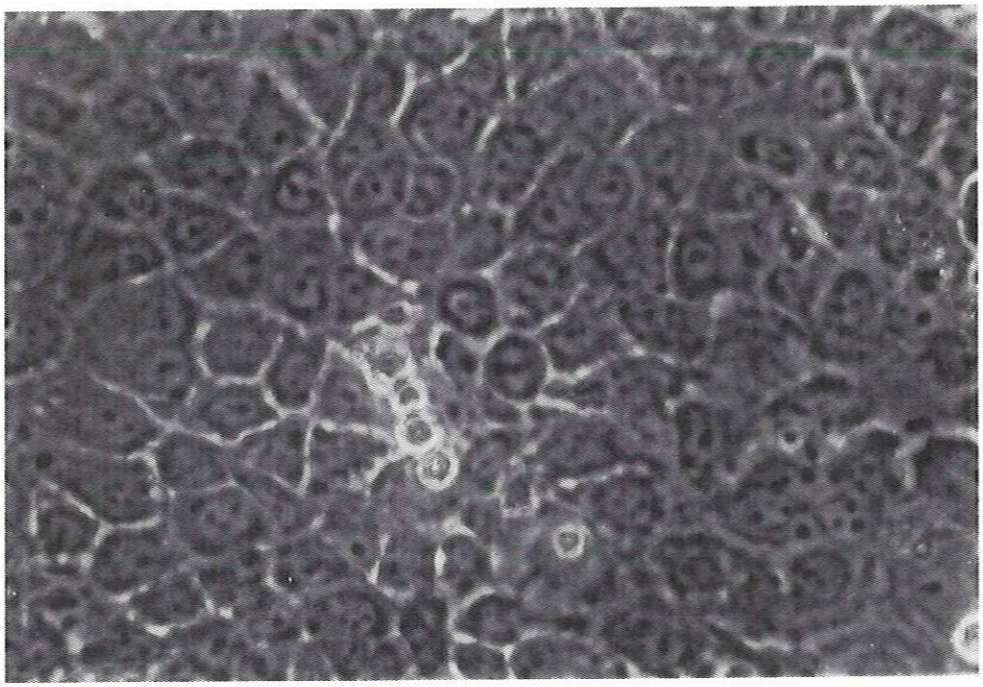


Fig. 7. Gerbil epidermal cells after 5 days in culture in low-Ca^{++} medium. Large intercellular spaces, presence of more than one nucleolus per cell and high nuclear-cytoplasmic ratio (×1200).

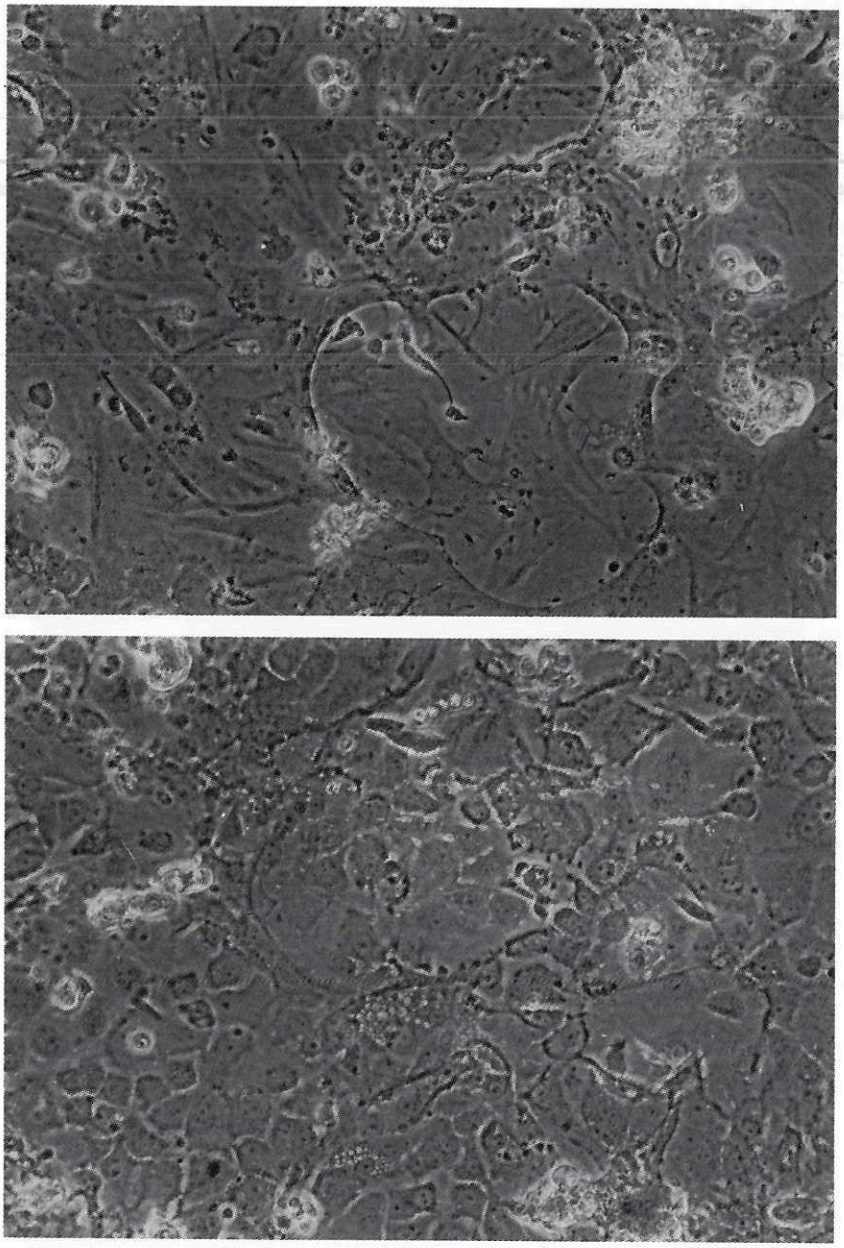


Fig. 8. Gerbil and mouse epidermal cells after one week in culture in low-Ca^{++} medium. (A) Mouse cultures, cells with large cytoplasm with irregular forms; (B) gerbil cultures, presence of vacuoloated cells (×480).

individually scattered in a sporadic manner and then tending to group together.

Gerbil epidermal cells in culture proceeded with a gradual stratification process: monolayer cells became larger and there was an increase in cell granulation, tonofibrillar networks could be recognized and cells with thickned membranes could be seen. Mouse cultures did not exhibit alterations (Fig. 11).

Gerbil cultures began to degenerate from the 45th day onwards.

Mouse cultures were observed for at least 2 months and during this period they persisted without alterations.

Effect of differential trypsinization

With the application of a modification of Yuspa's method, where a skin sample was utilized without separating dermis from epidermis, contamination with fibroblasts from the dermis was evident in the cultures. However, this contamination was never very intense, varying from culture to culture and in many situations not even being detectable. When this contamination did occur, with fibroblasts in large numbers, a differential trypsinization technique was used to remove the fibroblasts: when, on the other hand, small qauantities of fibroblasts appeared in culture, they were maintained in culture as they did not mix with epidermal cells and were restricted to the periphery of the culture, separate at first, but later forming bundles (Fig. 12).

The differential trypsinization technique was therefore applied in some experiments after 5 days in culture and 24 h later its results were already evident. Fibroblasts were removed without any sign of damage to epithelial cells. This technique also led to the elimination of the uppermost larger cells present in cultures in this phase, whereas the layer of basal cells still adhered to the substrate. These remaining cells began a new stratification process.

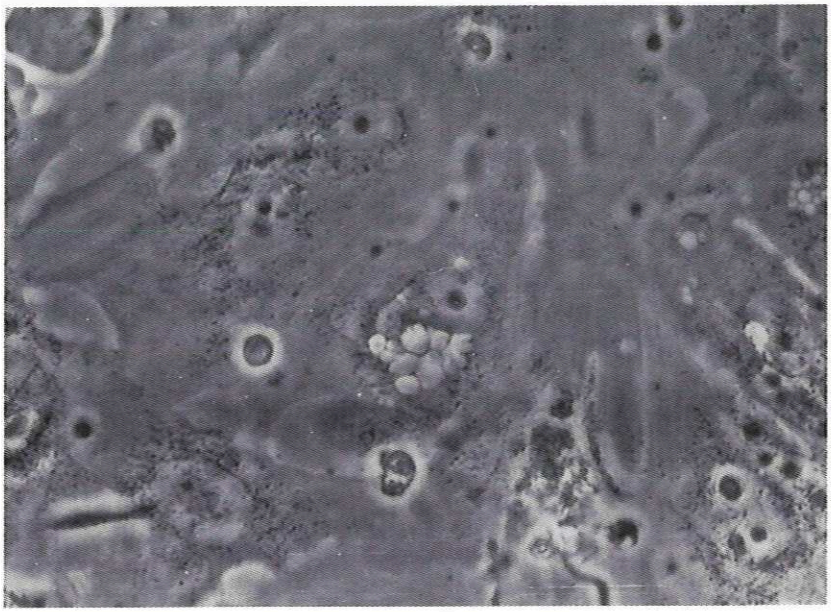


Fig. 9. Mouse epidermal cells after 9 days in culture in low-Ca^{++} medium. Presence of vacuolated cells and increased granulation (×1200).

Growth curve

The growth curves of gerbil and mouse primary epidermal cell cultures were studied under two temperature conditions and using two types of medium.

Gerbil epidermal cells presented two cycles of growth in cultures set up at 37°C (Fig. 13A): a multiplicative phase of 4 days, followed by a lag phase of 2 days, during which there was an abrupt decrease in the number of cells that incorporated thymidine. This was followed by a slow proliferative growth phase until the peak was reached on day 16, following which the number of cells that incorporated tritiated thymidine fell until day 21. The same, two-cycle pattern of proliferation occurred with cells incubated at 33°C (Fig. 13B), with cells reaching peaks of growth at approximately the same time points as cells incubated at 37°C. No important differences were observed that could be attributed to the presence or absence of calcium.

More noticeable differences could be observed in mouse epidermal cell cultures (Fig. 13C, D). Irrespective of temperature, the absence of calcium tended to cause a decrease in the proliferative rate from the second day of culture onward. The cultures at 37°C (Fig. 13C) in standard medium suggested a cyclic pattern, similar to that observed in gerbil cells, with an initial proliferative peak followed by a decline on the 4th day, and a subsequent slow rise until the 8th day when there was a new decline. Nevertheless, the cultures maintained at 33°C (Fig. 13D), after showing a small decline on day 4, stabilized on a plateau, followed by a decline on day 10.

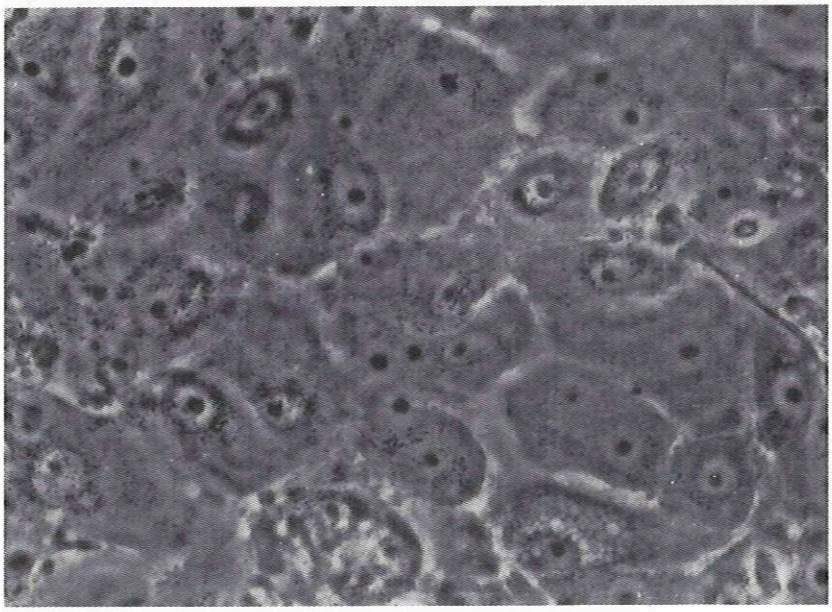


Fig. 10. Gerbil epidermal cells after 19 days in culture in low-Ca^{++} medium. Increased cellular granulation (×1200).

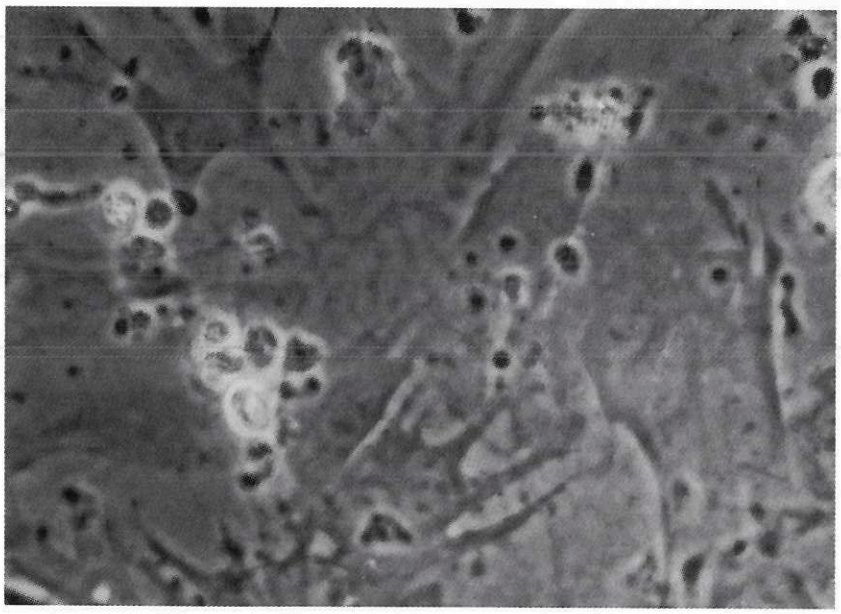


Fig. 11. Mouse epidermal cells after 47 days in culture in low-Ca^{++} medium (×1200).

DISCUSSION

The objective of the present work was to develop a technique for culturing gerbil epidermal cells and to compare such cells with those obtained by culturing mouse epidermis.

The usefulnes of any *in vitro* model depends on how much it resembles the *in vivo* model on which it is based. The gerbil cells obtained showed, after adhering to the substrate, an organization of basal epidermal cells that proceeded towards proliferation and differentiation. These morphological changes have been observed using phase-contrast microscopy and were further characterized by staining with HE, PAS, Sudan III and rhodanile blue.

The morphological characteristics corresponded in most respects to previously observed features in cultures of epidermal cells of other species and to what is seen of gerbil skin *in vivo*.

In the present study the first step in setting up the system constituted obtaining dissociated epidermal cells for plating in the cultures. In the first experiments based on the method of Yuspa et al. (7), only epidermis was used, and it proved difficult to obtain enough proliferative basal cells. In subsequent experiments, both dermis and epidermis were used, as described by Liu & Karasek (3). This method allowed the harvesting of proliferative basal cells but did not avoid contamination by fibroblasts, most epidermal cells adhered as clusters, forming islands, and this was the earliest indication of their epithelial nature. Above these islands, we could observe cell clumps, while isolated fibroblasts were seen between the

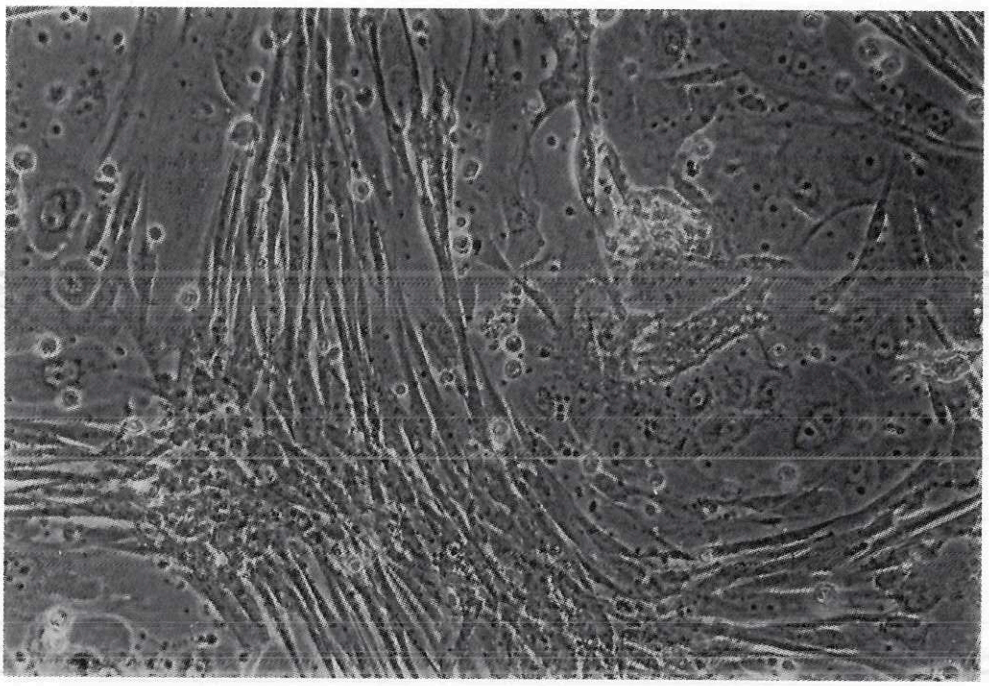


Fig. 12. Gerbil epidermal cells after 5 days in culture in standard medium. Fibroblasts forming bundles at the periphery (×480).

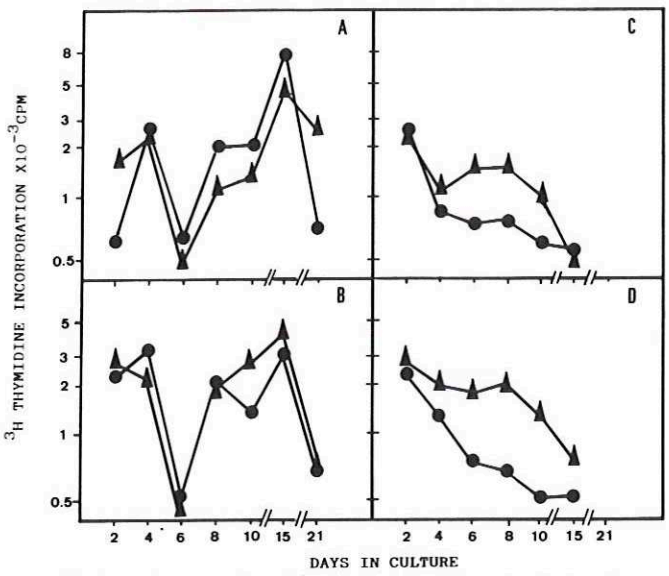


Fig. 13. Growth curve of gerbil and mouse epidermal cells in primary culture. (A) Gerbil epidermal cells grown at 37°C; (B) gerbil epidermal cells grown at 33°C; (C) mouse epidermal cells grown at 37°C; (D) mouse epidermal cells grown at 33°C. Cultures grown in medium with normal (▲) and low (●) Ca^{++} concentrations.

islands. When present in abundance, differential trypsinization (14) was used to eliminate these individuals. In this case, trypsinization was only performed after 5 days in culture, when some stratification had already occurred. The technique removed not only fibroblasts but also the superior layers of the epidermal cell culture, leaving only basal cells with proliferative capacity. These then started a new round of differentiation. This description agrees with Fusenig & Worst (16) who claimed that differentiation in cultures is not caused by epidermal cells already driven *in vivo* for keratinization, but by the maturation of basal cells *in vitro*.

There is considerable discussion regarding the possible need for a dermic element in epidermal cell cultures and whether they still maintain their capacity for growth and differentiation *in vitro* when they are deprived of mesenquima (2, 7, 17, 18). Because there is some evidence that fibroblasts may contribute to the growth of keratinocytes *in vitro* and because, even after trypsinization, slight fibroblast contamination persisted, it was preferred to consider these cultures in the way proposed by Jepsen et al. (19), as cultures with few fibroblasts rather than as cultures free of fibroblasts.

Another kind of cell present in the cultures was melanocytes, normally found in keratinocyte cultures of both mouse and gerbil skin. It was possible to observe dendritic cells full of microgranules, like pigmented melanocytes, involving keratinocytes and transferring their pigments to them. This has been described before (2, 20) and suggests an active interaction between the two cell types not yet clearly understood.

The epithelial cells forming islands *in vitro* resembled epidermal basal cells seen *in vivo*, in both morphology and proliferative capacity. When prliferation was measured with tritiated thymidine incorporation, a curve similar to that described in the literature on human epidermal cells (21) was seen. Growth was cyclic. There was an initial phase of proliferation during the first days in culture, followed by a pronounced decline leading to two new phases of growth. This cyclic conduct, with different proliferation peaks, is evidence of a normal process of maturation that leads to stratification and differentiation like the one observed *in vivo* (22) and reported by several authors *in vitro* (2, 6). A similar cyclic behaviour was also observed in mouse cultures maintained at 37°C in standard medium, although these cultures displayed a proliferative rate lower than that of gerbil cells. Similar results were also described *in vivo* (13), where the author suggested that the higher rate of proliferation found in gerbil epidermal cells reflects perhaps a more rapid rate of cellular revival, inherent to this animal species.

The first sign of stratification occurred with the initial polygonal cells increasing in size and connecting with each other by desmosomes; an intermediate layer consisting of intensely granular large cells was then visible. These granular cells stained with PAS and Sudan, suggesting the presence of a lipoglycoprotein complex. The presence of mucopolysaccharides in mouse epidermal cells has been described by other authors (23). The homogeneously distributed fine granular appearance was replaced by coarse, more fibrillar structures. As in the epidermis *in vivo*, these morphological changes *in vitro* occurred not in the basal layer of proliferating cells, but in a newly formed second layer. Subsequently, other morphologic alterations were also visible and vertical stratification took its natural course. The pattern of striation of cells developed further. Later the cells in the uppermost layer acquired a different morphology, similar to that of the cells of the stratum granulosum of the epidermis. The cell membrane thickened, nuclei became pyknotic and dense granulation appeared in the cytoplasm.

What was really striking in our cultures was the difference in behaviour between gerbil and mouse epidermal cells cultured in low-Ca^{++} medium. It is well known that the medium's Ca^{++} concentration regulates differentiation of human and mouse keratinocytes in culture (24, 25). Gerbil epidermal cells in culture also responded with morphological changes to low-Ca^{++} concentration, but this response was quite different when compared with the effect on mouse epidermis. Shortly after plating, mouse and gerbil epidermal cells showed similar morphology. Later, the two cell cultures started to display differences. Gerbil epidermal cells remained for a long time as small polygonal cells with a large nucleus, several prominent nucleoli and large intercellular spaces. This was followed by a slow and gradual stratification. Thus gerbil cells cultured in low-Ca^{++} medium differed not only from the presently observed cultured mouse cells in the same medium, but also from what has been reported in the literature when using mice, as the stratification of gerbil cultures was not inhibited despite being delayed. Mouse cells, besides showing a gradual decrease in proliferation from the 4th day onwards, showed irregular forms that persisted and maintained this morphology in culture for at least 2 months without stratifying. Cells with the characteristics of those shown here, with mouse cells in low-Ca^{++} medium, were also described by Yuspa & Harris (23) when they cultured mouse epidermal cells in presence of DMSO, and by Fischer et al. (26) with reference to mouse cell

lines cultured on 3T3 feeder layers and incubated at 37°C. Fischer et al. defined these cells as undifferentiated cells, whereas Yuspa & Harris called them quiescent cells. Even if it is still not clear to us how to classify them, the results from the proliferation curve suggest they are non-dividing quiescent cells.

It proved possible, therefore, to successfully grow gerbil epidermal cells *in vitro*, and these cells, when cultured in medium with low Ca^{++} concentration showed different characteristics to those of mouse epidermal cells in parallel cultures. This might reflect differences, already observed *in vivo*, between the skin of the two species, as well as their differing propensity to develop skin tumors. Gerbil epidermal cells cultures may prove a good model for the study of regulation of cell growth and differentiation.

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REFERENCES

1. Breidahl AF, Judson RT, Clunie GJA. Review of keratinocyte culture techniques: problems of growing skin. Aust NZJ Surg 1989; 59: 485–497.
2. Kitano Y. Keratinization of human epidermal cells in culture. Int J Dermatol 1979; 18: 787–796.
3. Liu Su-Chin, Karasek M. Isolation and growth of adult human epidermal keratinocytes in cell culture. J Invest Dermatol 1978; 71: 157–162.
4. Fusenig NE, Worst PKM. Mouse epidermal cell cultures. I. Isolation and cultivation of epidermal cells from adult mouse skin. J Invest Dermatol 1974; 63: 187–193.
5. Marcel CL, Kim YG, Kaine JL, Voorhees JJ. Stratification, specialization and proliferation of primary keratinocyte cultures; evidence of a functioning *in vitro* epidermal cell system. Cell Biol 1978; 79: 356–370.
6. Voigt WH, Fusenig NE. Organotypic differentiation of mouse keratinocytes in cell culture. A light- and electron-microscopic study. Biol Cell 1979; 34: 111–118.
7. Yuspa SH, Hawley-Nelson P, Stanley JR, Hennings H. Epidermal cell culture. Transplant Proc 1980; 12: 114–122.
8. Indo K, Wilson RB. Fetal rate keratinizing epidermal cells in culture: effects of long-term treatment by benzo[a]pyrene on their growth characteristics. J Natl Cancer Inst 1977; 59: 768–880.
9. Baden HP, Kubilus J. long-term culture of differentiating rat keratinocytes. J Invest Dermatol 1982; 78: 359.
10. Delescluse C, Cordier S, Frey J, Regnier M. Prunieras M. Studies on guinea pig skin cell cultures. Acta Derm Venereol (Stockh) 1978; 58: 1–7.
11. Regnier M, Delescluse C, Prunieras M. Studies on guinea pig skin cell cultures. I. Separate cultures of keratinocytes and dermal fibroblasts. Acta Derm Venereol (Stockh) 1973; 53: 241–247.
12. Liu Su-Chin, Karasek M. Isolation nd serial cultivation of rabbit skin epithelial cells. J Invest Dermatol 1978; 70: 288–293.
13. Guzman-Silva MA. *Análise morfológica comparativa da carcinogênese química cutânea entre camundongo (Mus musculus) e gerbil (Meriones unguiculatus)*. Niterói (Brazil), Universidade Federal Fluminense, Centro de Ciências Médicas, 1984 [M. Phil. thesis].
14. Milo GE, Moyses I, Donahoe J, Weisbrode S. Neoplastic transformation of human epithelial cells in vitro after exposure to chemical carcinogens. Cancer Res 1981; 41: 5096–5102.
15. Rheinwald JG, Green H. Formation of a keratinizing epithelium in culture by a cloned cell line derived from a teratoma. Cell 1975; 6: 317–330.
16. Fusenig NE, Worst PKM. Mouse epidermal cultures II. Isolation, characterization and cultivation of epidermal cells from perinatal mouse skin. Exp Cell Res 1975; 93: 443–457.
17. Bohnert A, Hornung J, Mackenzie IC, Fusenig NE. Epithelial-mesenchymal interactions control basement membrane production and differentiation in cultured and transplanted mouse keratinocytes. Cell Tissue Res 1986; 244: 413–429.
18. Madison KC, Swartzen D, Donald C, Wertz PW. Lamellar granule extrusion and stratum corneum intercellular lamellae in murine keratinocyte cultures. J Invest Dermatol 1988; 90: 110–116.
19. Jepsen A, MacCallum DK, Lillie JH. Fine structure of subcultivated stratified squamous epithelium. Exp Cell Res 1980; 125: 141–152.
20. Cruickshank CND, Harcourt SA. Pigment donation in vitro. J Invest Dermatol 1964; 42: 183–184.
21. Karasek MA. In vitro growth and maturation of epithelial cells from postembryonic skin. J Invest Dermatol 1975; 65: 60–66.
22. Matoltsy AG. Keratinization. J Invest Dermatol 1976; 67: 20–25.
23. Yuspa SH, Harris CC. Altered differentiation of mouse epidermal cells treated with retinyl acetate in vitro. Exp Cell Res 1974; 86: 95–105.
24. Hennings H, Michael D, Cheng C, Steinert P, Holbrook K, Yuspa SH. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. Cell 1980; 19: 245–254.
25. Hawley-Nelson P, Sullivan JE, Kung M, Hennings H, Yuspa SH. optimized conditions for the growth of human epidermal cells in cultures. J Invest Dermatol 1980; 75: 176–182.
26. Fischer SM, Viaje A, Harris KL, Miller DR. Improved conditions for murine epidermal cell culture. In vitro 1980; 16: 180–188.