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ISOLATION, CULTURE AND IMMUNOHISTOCHEMICAL IDENTIFICATION OF VAGINAL EPITHELIAL CELLS

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Abstract—Vaginal epithelial cells from 250-g Wistar rats were cultivated in plastic dishes without feeder layer support of cells of mesodermic origin. Isolated epithelial cells were obtained from everted vaginas by exposing the epithelium to a solution of 0.5% trypsin and cultivated in a modified Ham's F 12 medium supplemented with fetal calf serum, insulin, hydrocortisone and cholera toxin. In this way, epithelial cells gave rise to epithelioid colonies after 48 hr of culture. Infrequent fibroblast contamination was observed. Positive confirmation of the epithelial origin of the cell colonies was carried out with antikeratin antibodies and immunohistochemical techniques. This cell culture was shown to proliferate and differentiate *in vitro*. Proliferation has been assayed by the capacity of the cells to incorporate [^{3}H]thymidine and differentiation by the presence in the cell culture of cells possessing disulfide-bonded keratins and a chemically resistant protein envelope. Addition of 17β-estradiol to serum-deprived cell cultures induced an increase in the total protein per dish over the serumless cell cultures, but not reaching the values of serum-treated cultures. This tissue culture system seems to be a suitable model to study effects of hormones, growth factors etc. on proliferation and differentiation of isolated cells.

Key words: Vaginal epithelium, keratinocytes, tissue culture, keratins, immunocytochemistry

CULTURE, ISOLEMENT ET IDENTIFICATION IMMUNOHISTOCHIMIQUE DE CELLULES EPITHELIALES VAGINALES

Résumé—Les cellules épithéliales du vagin de rats Wistar de 250 g ont été cultivées dans des capsules de plastique en l'absence de la couche nutritionnelle d'origine mésodermique. Les cellules épithéliales ont été obtenues à partir de vagins éversés par l'exposition de l'epithélium à une solution de trypsine à 0,5%. Elles furent cultivées dans un milieu modifié de Ham F 12 en présence de sérum foetal de veau, d'insuline, d'hydrocortisone et de toxine de choléra. Après 48 hr de culture des cellules épithéliales on note l'apparition de colonies épithélioïdes. Elles ont été rarement contaminées par des fibroblastes. Par des techniques immunoshistochimiques avec un anticorps antikératine on a confirmé l'origine des colonies. On a démontré que la culture cellulaire prolifère et se différencie '*in vitro*'. On a évalué leur prolifération par la capacité des cellules d'incorporer la [^{3}H]thymidine et la différentiation par la présence dans la culture cellulaire de cellules possédant de la kératine stabilisée par des liaisons disulfures et une enveloppe protéinique chimiquement résistante. L'addition de 17β-oestradiol aux cellules privées de sérum induit une augmentation de la quantité totale de protéines par capsule sans arriver aux valeurs obtenues dans les cultures additionnées de sérum. Ce système de culture de tissu semble être un modèle approprié pour étudier les effets des hormones, facteurs de croissance etc. sur la prolifération et la différentiation des cellules isolées.

Mots-clefs: Epithélium vaginal, kératinocytes, culture de tissu, kératine, immunocytochimie

INTRODUCTION

The vaginal epithelium of rodents constitutes a useful model for the study of the effects of ovarian hormones on proliferation and differentiation of mammalian tissues (Clarke and Selye, 1942; Courrier, 1942; Eddy, 1966; Thrasher *et al.*, 1967). However, the complexity of the *in vivo* system and the possibility of multiple interactions between organs, tissues and even cell subpopulations, have hindered the complete understanding of the mechanisms of hormone action at cellular level. Although most of these problems can be at least partly overcome by using cultures of specific cell

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types, very few attempts have been made to obtain a reliable tissue culture system of the vaginal epithelium (Kahn, 1954; Flaxman *et al.*, 1973; Sobel *et al.*, 1979).

In the present paper, we describe a cell culture technique for vaginal epithelial cells of Wistar rats, starting from a monodisperse cell suspension. In addition we obtained a positive confirmation of the epithelial origin of this cell culture by using antikeratin antibodies and immunoperoxidase techniques.

MATERIALS AND METHODS

Isolation and culture of vaginal cells

Virgin female Wistar rats (250 g) were grouped according to their stage in the estral cycle. Rats in proestrus and diestrus were killed by a lethal inhalant dose of ether and vaginas were asceptically removed and rinsed in phosphate-buffered saline solution (PBS). Vaginas were dissected free of fat and connective tissue, and everted, exposing the epithelia to the dispersing solution. Everted vaginas were placed in 0.5% trypsin (Gibco) in PBS, pH 7.4 at 37°C, and incubated under continuous agitation. After 10 min, vaginas were decanted, the supernatant was discarded and fresh dispersing solution was added. The vaginas were incubated again at 37°C for 15 min and this time the supernatant was collected and the trypsin inactivated with bovine serum (Gibco). This last procedure was repeated 3 times or until the vaginas began to stick to each other, revealing that the vaginal surface was completely denuded of its epithelium. This was confirmed by histological sections. Cell suspensions were pooled, spun down at 500 *g* for 15 min and the cell pellet resuspended in culture medium. The medium used in this experiment was a Ham's F 12 modified according to Gerschenson *et al.* (1974) and supplemented with 10% fetal calf serum (Gibco), 10^{-5} M insulin (Lilly), 10^{-6} M hydrocortisone and 10^{-10} M cholera toxin (Sigma). Antibiotics were used in the following proportions: penicillin (600 i.u./ml), streptomycin (0.3 mg/ml) and amphotericin B (2 μg/ml). When indicated in the text, 17β-estradiol was added to a final concn of 10^{-7} M and, in those cases in which serum-free medium was used, 1 mg/ml bovine serum albumin (Sigma) was added. Cells were counted with an hemocytometer and platted at 8×10^5 cells per 25-cm^2 polystyrene Petri dish (Falcon). The culture medium was changed after 24 hr and after the first change it was replaced every other day.

Demonstration of disulfide-bonded keratin and cross-linked envelopes in cells was carried out by the technique described by Rheinwald (1980). Briefly, it consists of cell cytoskeleton and envelope resistance to 1% sodium dodecyl sulfate (SDS) and the same concentration of SDS plus 20 mM dithiothreitol.

In order to study proliferation, cell cultures were incubated with 2 μCi/ml [^{3}H]thymidine (20 Ci/mmole, New England Nuclear). After 24 hr of incubation the dishes were rinsed 3 times with PBS and fixed in methanol. Autoradiographs were carried out with Kodak NTB-3 emulsion according to the technique described elsewhere (Conti *et al.*, 1982). For the demonstration of total protein per dish, 2 ml of 1 N sodium hydroxide was added to each dish and left at room temp for 12 hr. Proteins were measured in an aliquot of the sodium hydroxide solution using the method of Lowry *et al.* (1951).

Antikeratin antibodies and immunohistochemical techniques

Keratin proteins were isolated from rat epidermis using a modification of the technique described by Sun and Green (1978). The epidermis was separated from the Wistar rat's foot pad by immersing the skin in water at 55°C for 1 min. Epidermal sheets were frozen and pulverized in liquid nitrogen with a mortar and pestle. Epidermal powder was extracted successively with 25 mM Tris buffer, pH 7.4, 1% Triton X and 8 M urea. Finally, insoluble proteins were extracted with 8 M urea plus 0.25 M 2-mercaptoethanol. The supernatant containing solubilized keratin polypeptides was dialyzed against water. Insoluble repolymerized keratin was spun down at 1000 *g*. Keratin obtained in this way was checked for purity by electrophoresis.

Antibodies against keratin were obtained in rabbits by injecting 0.5 mg of keratin in complete Freund's adjvant. Booster injections were given twice monthly with incomplete Freund's adjuvant. Sera from immunized rabbits were assayed by immunodiffusion according to the technique of Yen *et al.* (1976). The peroxidase–anti-peroxidase technique was carried out with positive sera using commercially available reactives (DAKO) (Sternberger *et al.*, 1970). Antikeratin serum adsorbed in keratin and preimmunized serum were used as control sera. Skin paraffin sections were used as a positive control of the immunohistochemical reaction. Cultures of 3T3 cells and fibroblasts were used as negative controls.

RESULTS

At the time of platting, epithelial cells appeared as single sphere-like cells or small clumps of two to four cells. Within 24 hr after platting, most of the cells were attached to the plastic surface and began gradually to spread out adopting colony-like morphology, characteristic of epithelial cells. After 48 hr cells were completely spread out (Fig. 1) but there were still clumps of cells on the top of the colonies. These clumps could be washed out by thoroughly pipetting with culture medium. Occasionally, contaminant fibroblasts could be seen but they were eliminated by careful timing of trypsinization. They can also be washed out with 0.02% EDTA solution as suggested by Rheinwald (1980).

The cultures showed proliferative capacity as shown by [^{3}H]thymidine incorporation into DNA by autoradiographs (Fig. 2). However, the number of attached cells increased slowly because a large number of cells were continually being shedded off into the medium. Since 95% of the cells released into the medium are resistant to SDS and 43% to the

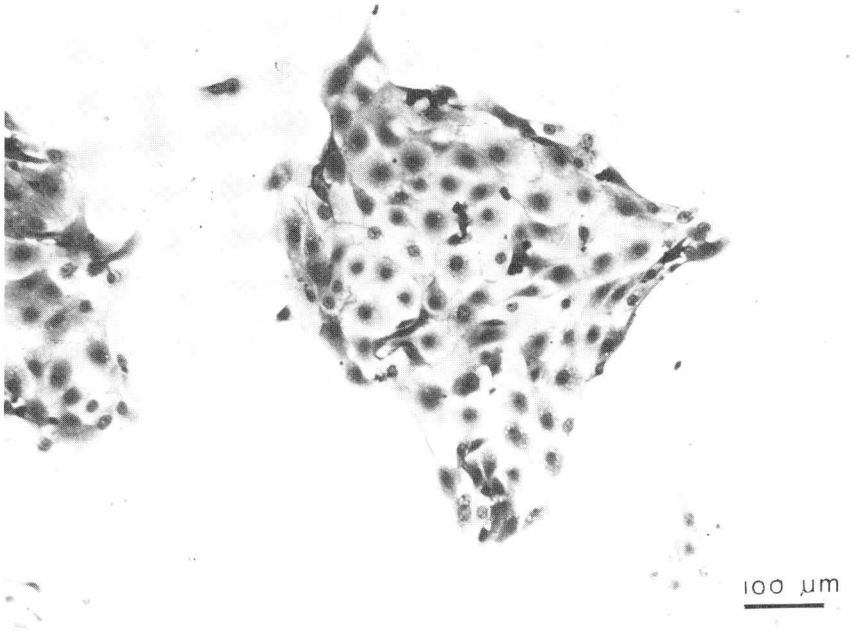

Fig. 1. Light micrograph of an epithelial cell colony after 3 days in culture. × 100 Giemsa.

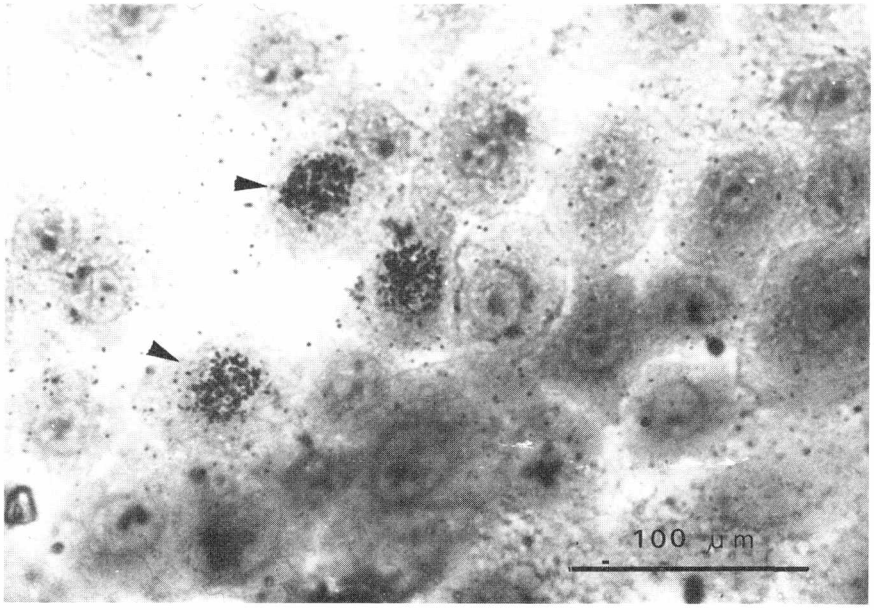

Fig. 2. Autoradiography of an epithelioid colony. Arrows show labeled cells. × 300 Giemsa.

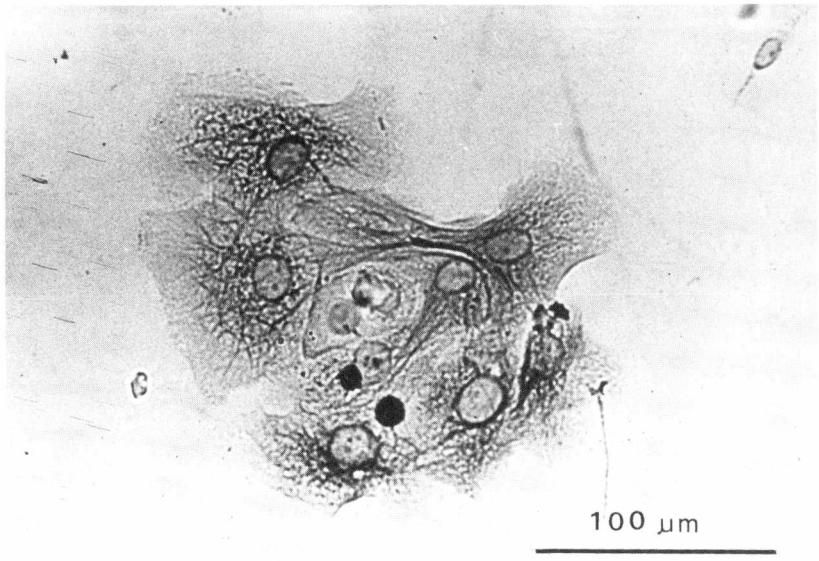

Fig. 3. Immunoperoxidase staining with antikeratin antibodies of an epithelioid colony. Filamentous arrangement of the keratin staining can be noticed. × 300.

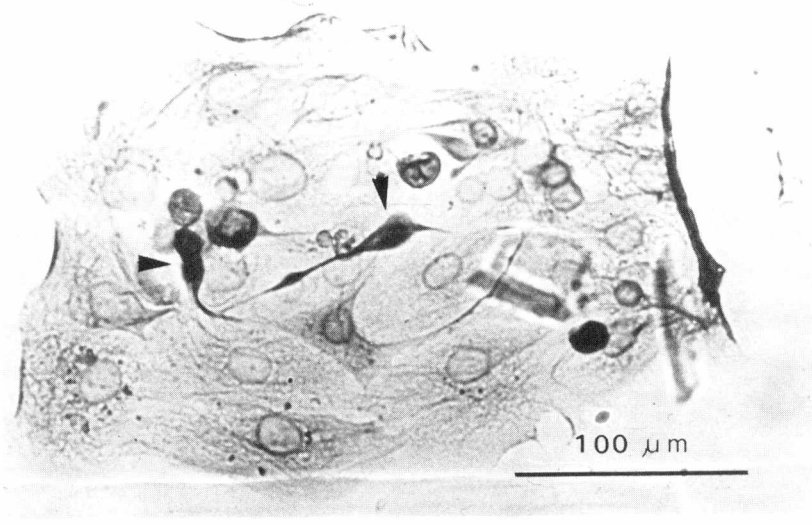

Fig. 4. Immunoperoxidase staining with antikeratin antibodies. Arrows show fusiform cell with intense reaction. × 300.

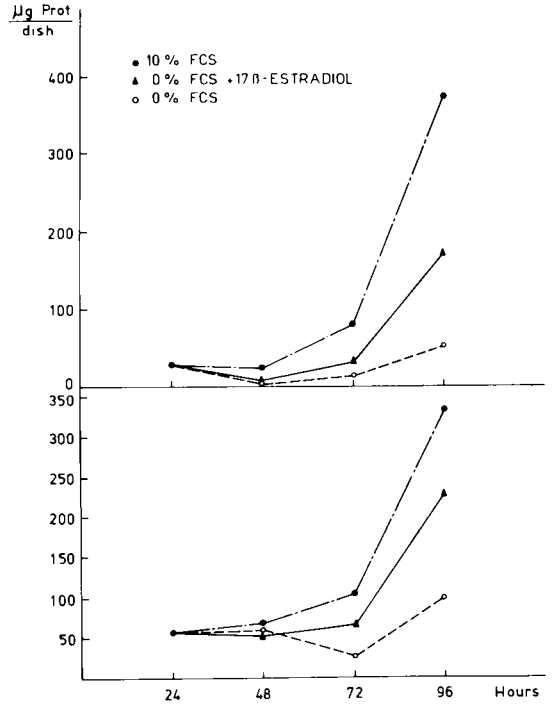

Fig. 5. Two independent experiments showing the response of vaginal cells to fetal calf serum (FCS) and 17β-estradiol. Each point represents the average of three Petri dishes.

combined effect of SDS and dithiothreitol, the process of cell shedding seems to be related to cell differentiation.

The immunochemical studies with antikeratin antibodies showed a positive reaction in the colonies. Higher magnification showed the characteristic fibrillar net of keratin of cultured epithelial cells (Fig. 3). On the other hand, cells floating in the medium had a very strong reaction. Fusiform cells, usually present on top of the epithelial colonies, also gave a strong reaction (Fig. 4).

In order to study the response of this culture model to growth factors and hormones, the following experiment was carried out: 24-hr-old cultures were tested under three different conditions: serum-free medium, serum-free medium supplemented with 17β-estradiol and medium with 10% fetal calf serum. Results of two independent experiments are shown in Fig. 5. It can be seen that both fetal calf serum and 17β-estradiol increase the total protein per dish.

DISCUSSION

We have described a simple method for the culture of monodisperse cells from the vaginal epithelium of Wistar rats. Previous attempts have been made to perform cultures of the vaginal epithelium, but in all these cases explant techniques were used (Kahn, 1954; Flaxman *et al.*, 1973; Sobel *et al.*, 1979). Other Malpighian epithelia have received more attention; for example Rheinwald and Green (1975) developed a reproducible method for the establishment of proliferating human foreskin keratinocytes on a lethally irradiated feeder layer. This technique has been used successfully by many authors (Miller *et al.*, 1980; Steinberg and Defendi, 1979; Kodo *et al.*, 1979). However, since our main purpose is to study the direct effect of ovarian hormones on the cultured epithelial cells, avoiding either putative indirect effects or interactions between different cell types (Sonnenschein and Soto, 1980; Murai *et al.*, 1980), the aim of the present work was to obtain a cell culture system of vaginal epithelial cells without any participation of mesodermal components, which is not the case in explant or feeder layer techniques.

We have isolated vaginal cells following a technique used by Gerschenson *et al.* (1974) for the culture of rabbit uterine hormones, also using Gerschenson's modification of Ham's F 12 medium. However, this medium was supplemented with cholera toxin and hydrocortisone which have been shown in keratinocyte cultures to prevent terminal differentiation, and to inhibit stratification and have also been suggested to prevent the lost of epithelial morphology (Rheinwald, 1980). However, when using these cultures in the study of hormones or other promoting growth factors, the addition of insulin, hydrocortisone and cholera toxin should be carefully checked for possible interaction with the hormone under study.

Vaginal cells cultured in this way have been shown to proliferate and differentiate *in vitro*. Proliferation has been assayed by the capacity of the cells to incorporate [^{3}H]thymidine and we have considered the presence of disulfide-bonded keratins and a chemically resistant protein envelope as a marker of cell differentiation (Rheinwald, 1980). Most of the cells shedded off to the culture medium have been shown to be resistant to SDS and 2-mercaptoethanol which proves differentiation in this system.

In order to work with specific cell types, it is very important to be absolutely certain of the origin of cultured cells and usually appropriate markers should be used to confirm the origin of these cell types. In recent years Franke *et al.* (1978) and Sun

and Green (1978) have shown that keratin proteins are not restricted to keratinized epithelium, but are also present in most of the covering epithelia. Several papers have shown that keratins are also present in cell cultures and tumours derived from epithelial tissues (Franke *et al.*, 1978; Sun and Green, 1978; Schelegel *et al.*, 1980; Gabbiani *et al.*, 1981) and another paper has shown keratin filaments in very early stages of embryo development (Paulin *et al.*, 1980). In consequence, keratin proteins can be considered to be a reliable marker to demonstrate epithelial origin. Here, we have used an immunoperoxidase technique against keratin to obtain positive confirmation of the epithelial origin of our cell colonies. In the same way have also been able to show that fusiform cells present on top of the epithelial colonies are not migratory fibroblasts but epithelial cells undergoing terminal differentiation and in the process of being sloughed off.

The cell culture described here has proved to be responsive to estrogens and fetal calf serum. It seems a useful model to study the direct effect of ovarian hormones and other factors (promoting agents, chemical carcinogens etc.) on the vaginal epithelium, since it is a cell culture with a confirmed epithelial origin and it has the capacity of proliferation and differentiation *in vitro*.

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REFERENCES

Clarke, E. and Selye, H., The action of steroid compounds on the vaginal epithelium of the rat. *Am. J. med. Sci.* **204,** 401–409 (1942).

Conti, C. J., Aldaz, C. M. and Gimenez, I. B., A simple procedure to perform autoradiographies in keratinocyte cultures. *Stain Technol.* **57,** 355–357 (1982).

Courrier, R., L'action keratinizante de la desoxycortiscosterone sur le vagin, et les different propietes de trois substances hormonales progestatives. *C. r. Séanc. Soc. Biol.* **136,** 196–198 (1942).

Eddy, E. M., Differentiation of vaginal epithelium under hormonal influence as revealed by fine structure. *Anat. Rec.* **154,** Abstr. 342 (1966).

Flaxman, B. A., Chopra, D. P. and Newman, D., Growth of mouse vaginal epithelial cells *in vitro*. *In Vitro* **9,** 194–201 (1973).

Franke, W. W., Schmid, E., Osborne, M. and Weber, K., Different intermediate-sized filaments distinguished by immunofluorescence microscopy. *Proc. natn. Acad. Sci. U.S.A.* **75,** 5034–5038 (1978).

Franke, W. W., Weber, K., Osborn, M., Schmid, E. and Freudenstein, C., Antibody to prekeratin: decoration of tonofilament-like arrays in various cells of epithelial character. *Expl Cell Res.* **116,** 429–445 (1978).

Gabbiani, G., Kapancy, Y., Barazzone, P. and Franke, W. W., Immunochemical identification of intermediate-sized filaments in human neoplastic cells. *Am. J. Path.* **104,** 206–216 (1981).

Gerschenson, L. E., Berliner, J. and Jing-Jing Yang, Diethyl-stilbestrol and progesterone regulation of cultured rabbit endometrial cell growth. *Cancer Res.* **34,** 2873–2880 (1974).

Kahn, R. H., Effect of estrogen and of vitamin A on vaginal cornification in tissue culture. *Nature, Lond.* **174,** 317 (1954).

Kodo, S., Aso, K. and Namba, M., Culture of normal human epidermal cells with 3T3 feeders on millipore filters. *J. invest. Derm.* **72,** 85–87 (1979).

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265–275 (1951).

Miller, D. R., Hamby, K. M., Allison, D. P., Fischer, S. M. and Slaga, T. J., The maintenance of a differentiated state in cultured mouse epidermal cells. *Expl Cell Res.* **129,** 63–71 (1980).

Murai, J. T., Conti, C. J., Gimenez-Conti, I. B., DePaoli, J. R., Conner, E. A. and Gerschenson, L. E., Hormonal regulation of proliferation and differentiation of rabbit uterine epithelium: cell culture and animal studies, in: *The Endometrium*, Kimball, F. A. (ed.). SP Medical and Scientific Books, New York (1980).

Paulin, D., Babinet, C., Weber, K. and Osborn, M., Antibodies as probes of cellular differentiation and cytoscheletal organization in the mouse blastocyst. *Expl Cell Res.* **130,** 297–304 (1980).

Rheinwald, J. G., Serial cultivation of normal human keratinocyte, in: *Methods in Cell Biology*, Vol. 21, Harris, C. C., Trump, B. F. and Stoner G. D. (eds), pp. 229–254. Academic Press, New York (1980).

Rheinwald, J. G. and Green, H., Serial cultivation of strains of human epidermal keratinocytes. The formation of keratinizing colonies from single cells. *Cell* **6,** 331–344 (1975).

Schlegel, R., Banks-Schlegel, S., McLeod, J. A. and Pinkus, G. S., Immunoperoxidase localization of keratin in human neoplasms. *Am. J. Path.* **101,** 41–50 (1980).

Sobel, J. D., Tchao, R., Bozzola, J., Levison, M. E. and Kaye, D., Human vaginal epithelial multilayer tissue culture. *In Vitro* **15,** 993–1000 (1979).

Sonnenschein, D. and Soto, A. M., But are estrogens per se growth promoting hormones? *J. natn. Cancer Inst.* **64,** 211–215 (1980).

Steinberg, M. L. and Defendi, V., Altered pattern of growth and differentiation in human keratinocytes infected with simian virus 40. *Proc. natn. Acad. Sci. U.S.A.* **76,** 801–805 (1979).

Sternberger, L. A., Hardy, P. H., Cuculis, J. J. and Meyer, H. G., The unlabeled antibody enzyme method of immunochemistry. Preparation and properties of soluble antigen–antibody complex (horseradish peroxidase) and its use in identification of spirochetes. *J. Histochem. Cytochem.* **18,** 315–333 (1970).

Sun, T. and Green, H., Immunofluorescence staining of keratin fibers in cultured cells. *Cell* **14,** 469–476 (1978a).

Sun, T. and Green, H., Keratin filaments of cultured human epidermal cells. *J. biol. Chem.* **253,** 2053–2060 (1978b).

Sun, T., Shih, C. and Green, H., Keratin cytoskeleton in epithelial cells of internal organs. *Proc. natn. Acad. Sci. U.S.A.* **76,** 2813–2817 (1978).

Thrasher, J. D., Clark, F. I. and Clarke, D. R., Changes in the vaginal epithelial cell cycle in relation to events of the estrous cyclus. *Expl Cell Res.* **45,** 232–236 (1967).

Yen, S. H., Dahl, D., Schachner, M. and Shelanski, M. L., Biochemistry of the filaments of the brain. *Proc. natn. Acad. Sci. U.S.A.* **73,** 529–533 (1976).