Separation Methods of Epitheloid and Fibroblastic Cells in Tissue Culturing of Peritoneal Exudates of Mink

*Zensaku YOSHII and George A. PADGETT

Department of Veterinary Pathology, College of Veterinary Medicine, Washington State University Pullman, WASH. 99163

Keith R. FARRELL

Endoparasite Vector Pioneering Research Laboratory U. S. D. A., Pullman, WASH. 99163

(Received May 31, 1972)

SUMMARY

Usefulness and Necessity of separation of epitheloid and fibroblastic cells in tissue culturing were emphasized. Two methods with several techniques were examined by using mink cells from peritoneal exudates. Harvesting method of necessary cells or colonies contains three techniques. They are punching for both epitheloid and fibroblastic cells, slight digestion with ATV-solution for fibroblastic cells mainly, and scratching for fibroblastic cells. Elimination method of unnecessary cells or colonies contains two techniques. They are erasing (almost same as scratching) and slight digestion. The methods with these techniques have been proven practical for separation. Finally, a systematic figure of the technical procedures and methods was established by the authors. Selection of suitable flasks, adoption of adequate technique and method, combination of techniques, and repetition of techniques are recommended for routine work in the separation of one type cell. Efficiency and purity of the results are also discussed.

INTRODUCTION

In tissue culture of mink cells harvested from peritoneal exudates, several types of cells are observed in culture flasks. These cells are classified into three major groups: epitheloid, fibroblastic, and intermediate types.

It is very clear that before cloning of these cells, separation of each type is more advantageous. Even if cloning is not desired, a pure culture of the separated cell strain is more useful than a mixed culture, not only for the study of the cell

*Present Address: Department of Microbiology, Yamaguchi University School of Medicine Ube, Yamaguchi-ken, JAPAN (755)

strain itself, but also for the application of pathogenic microorganisms.

Some mechanical and chemical separation methods, such as the use of a plasma coagulum,[1)2)3)] differential centrifugation or gradient sedimentation in sucrose or albumin,[4)] electrophoresis of different class cells,[5)] and inhibition of other type of cells than epitheloid cells,[6)7)8)] are well known at the present time. However, these methods are not always suitable for every kind of cell, but are somewhat complicated and difficult, and sometimes yield poor results ; simpler methods are needed.

Therefore, some separation methods were attempted using the cultural characteristics of these cells with various physical and chemical treatments. Some of them proved successful, and are reported in this paper.

MATERIALS

1. Mink
   a. Chediak-Higashi Syndrome[9)] ..... 11 animals
   b. Non Chediak-Higashi Syndrome..... 11 animals
2. Culture flasks and tubes
   a. Falcon plastic flasks..... 30 ml and 250 ml
   b. Leighton tubes..... Bellco Glass, Inc.
3. Digestive enzyme
   ATV-solution[10)] ..... 1/2×, 2/3×, 1×, 1.5×
4. Culture media
   a. Basic media : 199, NCTC-109, McCoy, MEM
   b. Sera : Calf serum, Fetal calf serum (FCS)
5. Special instruments and materials
   a. Rubber policeman (self-made)..... see Photo. 1
   b. Cork punch
   c. Forceps (long and short)
   d. Other items : Red and black china markers, platina loop, 70% Ethanol, cotton balls, parffin oil-Ringer's solution mixture (1 : 4)

METHODS

Over a one-year period many preliminary experiments were repeated to determine suitable conditions for technical procedures. The results obtained from each technical procedure are summarized in the following paragraphs.

Harvesting of cells : Four or five days after intraperitoneal injection of paraffin oil-Ringer's solution mixture, mink peritoneal exudate is drained by reflux of

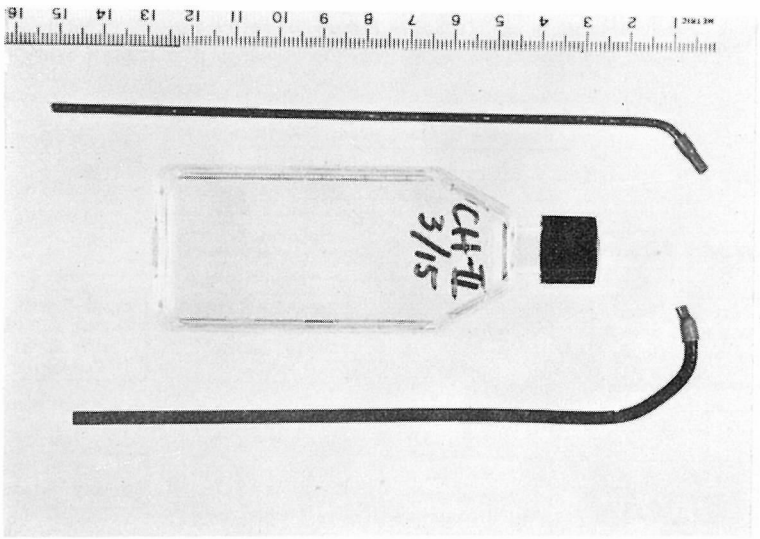


Photo. 1. Falcon Plastic Flask (30 ml) and Self-made Rubber Policeman (2 pieces)

Ringer's solution. Cells are then harvested by centrifugation at 1,000 r.p.m. for 5 minutes, and prepared in a final cell suspension of Ringer's solution in the number 10^5 to 10^6 per ml. The majority of cells in this suspension were macrophages.

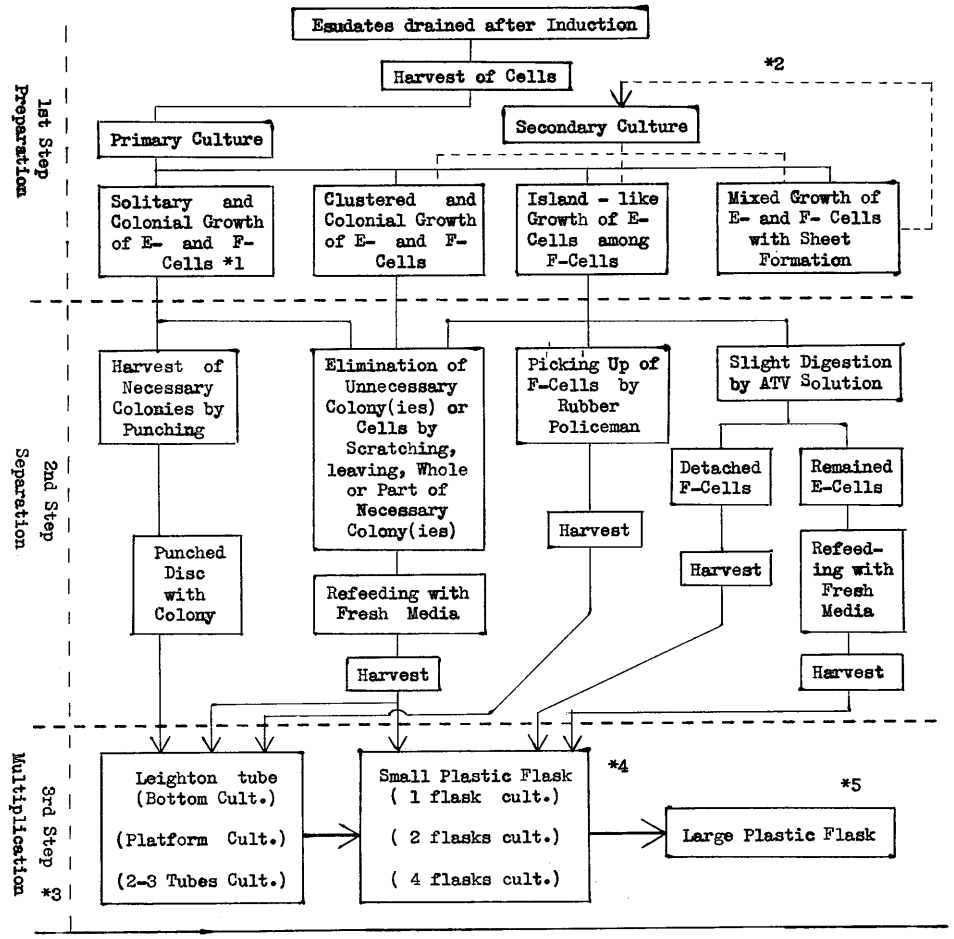
Primary culture : One-half ml of the cell suspension described above is inoculated into a Falcon plastic flask (30 ml) containing 4.5 ml of fresh media and incubated under a closed system at 37° C. When cultivation is successful, a number of small colonial growths or dispersed growths of cells appear within 7 to 14 days. The former situation is often seen with a dilute inoculum of less than 5×10^5 cells, and the latter with a more concentrated inoculum of over 10^6 cells. Media change of the flasks was accomplished every 4 days during incubation periods.

Subculturing : In cell transfer, multiplication is usually made to between 1.5 and 3 times the original flask number. After digestion with ATV solution, cells detached from the flask wall are harvested by centrifugation at 500 r.p.m. for 5 minutes, and transferred into new flasks containing fresh media. In subculturing, epitheloid cells usually show an island-like growth, surrounded by fibroblastic cells showing dispersed growth in a whirl arrangement. Fibroblastic cells grow faster than epitheloid cells in general.

Intermediate form of cells were relatively very few in general ; these were not considered to be important.

During repeated subculturing, several separation techniques were attempted on suitable flasks with good cell growth.

Fig. 1. Systematic Figure of the Technical Procedures in the Separation Methods of Epitheloid and Fibroblastic Cells from the Peritoneal Exudates of Mink



*1 E-Cells ..... Epitheloid Cells, F-Cells ..... Fibroblastic Cells
*2 Repeat the same technical procedures
*3 Multiplication of the separated cells is depended upon the number of applied cells
*4 & *5 Falcon Plastic Flasks (30 & 250 ml)

PROCEDURE OF BASIC SEPARATION METHODS

The separation methods which established by the authors are summarized in Figure 1. The system consists of three major steps ; the first step is the technical procedure for the preparation of cells, the second step is the separation procedure, and the third step is the stage of multiplying and subculturing of the separated cells. Procedures of the system are explained in detail as follows :

Cell conditions

Media : Many kinds of media were tested preliminarily. As basic media, Medium-199 and MEM, with the addition of calf serum or fetal calf serum in the concentration of 20 %, are suitable for good growth of both fibroblastic and epitheloid cells. MEM with 20 % fetal calf serum is effective in multiplying epitheloid cells even in mixed cultures. Therefore, these media and sera were employed for separation and subculturing. Maintenance of epitheloid cells in culture after separation is sometimes difficult. For that case, Medium-199 with 20 % fetal calf serum decanted from fibroblastic cells was added to an equal part of fresh media (usually MEM with 20 % fetal calf serum) and used for feeding of the epitheloid cells.

Growth situations : In successful primary cultures, solitary colonial cell growth or mixed colonial growth can be seen at the bottom of the flasks.

Flasks with solitary or clustered colonial growth of one type of cell in the primary culture and island growth of epitheloid cells among fibroblastic cells in the maintenance culture are suitable for the separation.

Flasks with dispersed and mixed growths of epitheloid and fibroblastic cells are used for subculturing.

Although the cells which appear in the culture flasks show a variety of types, they are classified into three major groups as described in the introduction. Epitheloid and fibroblastic cells are in the major, while a third group, the intermediate form, for example amoeba type, is less frequently seen. In this study, epitheloid and fibroblastic cells are regarded as the main targets for the separation. The third type is very difficult to separate from pure cells of the other types. This phenomenon will be discussed later from the viewpoint of transformation.

In secondary and later cultures a monolayer growth of cells of mixed types is usually seen. The growth situations of epitheloid and fibroblastic cells in subcultures show somewhat different aspects of phase and speed. Fibroblastic cells usually grow and degenerate faster than epitheloid cells. Epitheloid cells sometimes show colonial, island-like growth surrounded by fibroblastic cells with whirl-like growth. These facts are regarded as useful characteristics for the separation.

Treatment of Cells of Each Type

Harvesting methods of necessary cells or colonies

1. **Punching technique.** Colonial growth areas are circled with a red china marker on the outer surface of the plastic flask under inverted microscopy ; the above areas are remarked by scratching with the sharp point of a metal instrument overlapping the same circle line. Then the flask is filled with Ringer's solution to almost 80% capacity. Erasing of the red circle line and disinfection of the outer surface of the flask are then accomplished with 70% ethanol. Next the circled area showing the location of colonial cell growth is punched from the flask with a flamed and heated punch (Photo. 2).

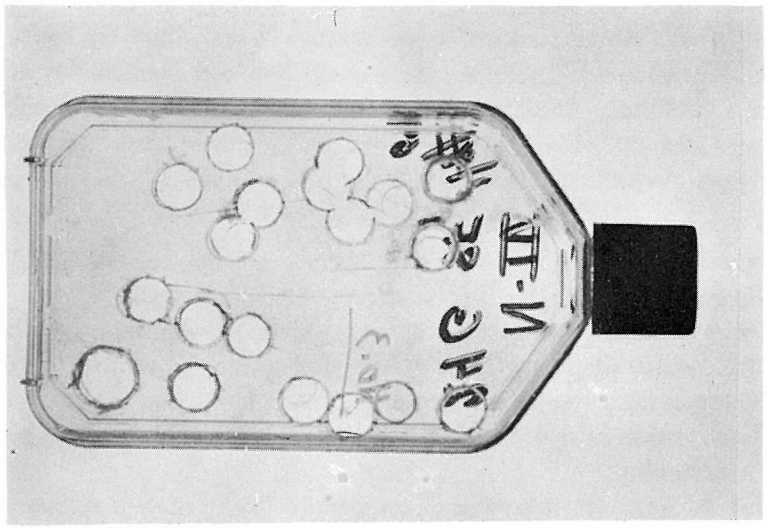


Photo. 2. Falcon Plastic Flask (250 ml) with many Punched Holes

A small disc containing one cell type colony is now obtained (Photo. 3) which is transfered into a Leighton tube containing fresh media (the diameter of the punched disc must be smaller than that of the Leighton tube). After 2 or 3 days of incubtion at 37°C, ATV-solution is added to detach the cells from the disc, and the disc is removed from the tube. Then cells were harvested by centrifugation at 500 r.p.m. for 5 minutes, cultured at the bottom of the tube with additional fresh medium, and incubated at 37°C for about 2 weeks. Medium change was made 3 or 4 times during this period.

After confirmation of good growth of a single type cell on the tube bottom, cells were detached with ATV-solution and reseeded by refeeding on the platform of the Leighton tube. Use of the same Leighton tube is convenient as a technical

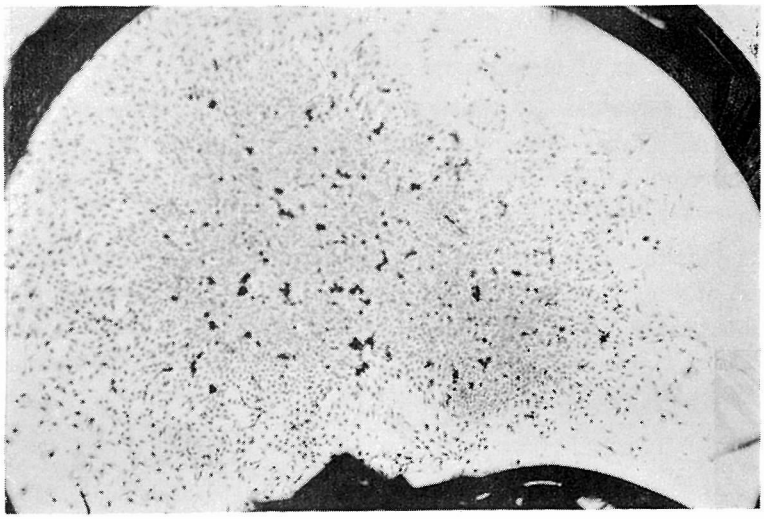


Photo. 3. Punched disc with a colony of Epitheloid cell type

procedure and useful for cell multiplication from the viewpoint of population size. Cell numbers can be gradually increased, for example, from one Leighton tube to 2, from 2 to 4, and from 4 to one small Falcon plastic flask. Thus sufficient numbers of a single type of cell from one colonial growth can be obtained.

2. **Slight digestion technique for Fibroblastic cells.** Although ATV-solution is effective for the detachment of all cell types, some differences can be seen between its action on epitheloid and fibroblastic cells. In general, epitheloid cells are more resistant than fibroblastic cells. Particularly in aged mixed cultures containing both types of cells, the latter usually comes off the flask wall faster than the former. This fact is useful in separation, especially for harvesting fibroblastic cells. One-half or two thirds times the concentration of ATV-solution can be used for slight digestion in order to harvest fibroblastic cells. If the digestion is done somewhat strongly, most of the fibroblastic cells come off, and most of the epitheloid cells still remain. The epitheloid cells can then be obtained by ordinary ATV digestion. Thus the slight digestion technique can be used to harvest either type of cell.

3. **Scratching technique (for picking up).** As fibroblastic cells survive scratching and removal from the flask wall by a rubber policeman better than epitheloid cells, an area of fibroblastic cells can be scratched by this method. Clumps of fibroblastic cells come off the flask wall and can be harvested by low centrifugation at 500 r.p.m. for 3 minutes with subsequent transferral into new flasks or Leighton tubes. Some of the scratched cells survive and grow after several days' incubation at 37°C. The surviving cells are then transferred and multiplied as a

pure culture.

Elimination methods of unnecessary cells or colonies

1. **Scratching technique for Erasing.** This is the same technique as the scratching above described. The scratching technique can be used to eliminate unneces sary cells or colonies. The necessary cells remaining in the flask can then be refed with fresh media.

2. **Slight digestion technique for Fibroblastic cells.** In general, this is used for elimination of fibroblastic cells and for maintenance of epitheloid cells in one flask. After elimination of fibroblastic cells by slight digestion with ATV, epitheloid cells can be refed with fresh media. After several days' incubation, epitheloid cells can be harvested and transferred.

Practical Use and Application

For harvesting, the objective cells must be more abundant than other types of cells. Flasks with this situation are suitable for separation because unnecessary colonies or cells can be eliminated easily, and necessary colonies or cells can be harvested. The growth situation of each type of cell is very important ; aggregate growth of each type is more efficient. A suitable flask must be chosen, and the greater the number of suitable flasks, the more efficient the harvesting and the separation.

When the growth situation is good, three separation techniques are available above described. (If the growth situation is poor, subculturing is necessary).

A combination of these techniques is sometimes recommended. For example, the punching is very good for solitary colonial growth of a single type of cell ; but when epitheloid cells grow in colonial form and are surrounded with fibroblastic cells, elimination by scratching with a rubber policeman or slight digestion is necessary to isolate the desired colony. The punching may then be employed. On the other hand, in the case of abundant growth of fibroblastic cells containing some epitheloid cells in island-like growth, scratching of the epitheloid cells is adequate to eliminate them ; ATV treatment can then be used for the harvest of fibroblastic cells.

One separation method is not always successful for complete separation of epitheloid and fibroblastic cells. If incomplete separation occurs an attempt can be made with anothers' technique, whether alone or in combination with other methods. If subculturing of separated cells is unsuccessful, the experiment must be repeated until successful separation is obtained.

Indications of Cells for Technical Procedures

When solitary colonies are cultured in the primary stage with single colonies

of one type of cells in each flask, the flask may be incubated with appropriate media change until growth becomes sufficient for transfer.

If some unneccessary colonies exist, elimination by scratching with rubber policeman is recommended ; the remaining colonies can be grown and passed as usual.

Punching of the necessary colonies and transfer of them into Leighton tube are also available.

Then the maintenance cultures of single-type cells can be continued.

If solitary and clusterd colonies with mixed cells appear in one flask, eliminate the latter by scratching with a rubber policeman. Solitary colonies remaining can be maintained by feeding. If growth is good, single type of cells can be transferred. When colonies of different cell types appear mixed together in one flask, eliminate all but part of neccessary colonies, maintain the remaining parts, and tranfer them.

On a cell sheet with a mixed situation, separation should not be attempted. Transfer cells and await the next opportunity following the growth of secondary cultures.

In the secondary and maintenance cultures a definite colonial and island-like growth of epitheloid cells should occur among fibroblastic cells. If the slight digestion technique is successful, fibroblastic cells can be harvested and subcultured. However, a 100% pure culture of fibroblastic cells is sometimes difficult to obtain after one digestion. Therefore, repetition of the slight digestion technique, especially with aging cultures, is recommended. Epitheloid cells are very easily erased with a rubber policeman. Therefore, the elimination of epitheloid cells by erasing is recommended ; fibroblastic cells remain and can be harvested.

When a completely mixed growth of epitheloid and fibroblastic cells forms a cell sheet, separation should not be attempted. Transfer the cells after diluting the harvested suspension, and await the good condition for separation in the next generation. Following good growth situations, the methods above mentioned will be useful.

RESULTS AND DISCUSSIONS

Punching technique in primary culture

Results are shown in Table 1. The punching technique was not difficult to excute ; however, the subculturing and multiplying of separated cells were very difficult. Particularly in the case of epitheloid cells there was no success. In the case of fibroblastic cells, only three successful cases were obtained in 20 experiments. The punching is regarded as the most suitable for precloning, as it separates

Table 1. Results of Punching technique in Primary Cultures

	Epitheloid cells	Fibroblastic cells
Success of Punching	20/20	20/20
Success of Subculturing	0/20	3/20

Numerator ..... Successful results
Denominator ..... Experimental cases

almost pure colonies. However, the results were so poor that this technique is not considered practical for separation. If many colonial growths of necessary cell types are punched and, gathered up then perhaps some more successful results can be expected in the subculturing.

Although the causes of failure in the subculturing of punched epitheloid cells are not clear, ATV-treatment of the cells on the discs, low population in one Leighton tube, and poor media for multiplying are regarded as the important problems. As epitheloid cells are usually resistant to ATV-treatment, particularly in aging cultures (more than 3 weeks of age), detachment from the disc wall with transfer into the Leighton tubes was very difficult. Even when harvest and transfer were successful, the population number in each Leighton tube was very low. Therefore, large colonies in young cultures would probably be more successful. Concerning media, higher concentration of serum, such as 30–35 % fetal calf serum in MEM medium might be recommended.

Slight digestion technique with ATV-solution

Results of this technique applied to the island-like growth of epitheloid cells among fibroblastic cells are shown in Table 2. The digestion effect is obtained with $1/2\times$ or $2/3\times$ concentration of ATV applied for between 10 and 30 minutes (time varies with cells and flasks). Most of the fibroblastic cells detach from the flask wall, and the epitheloid cells usually remain. Therefore, the fibroblastic cells can be harvested, and remaining epitheloid cells are refed with fresh media. Although separation was accomplished by this technique, complete separation was still difficult. Some epitheloid cells intermingled with the detached cells, and some fibroblastic cells remained in the flask with epitheloid cells. In order to obtain a greater number of pure fibroblastic cells, digestion should be done with a lower ATV concentration for a shorter time. For obtaining epitheloid cells, digestion should be done with a higher ATV concentration for a longer time ; or digestion may be repeated as necessary after refeeding.

When subculturing was repeated after the slight digestion technique, transferred fibroblastic cells formed pure colonies ; but the flask with the epitheloid cells sometimes showed revived fibroblastic cells among the epitheloid cells. Moreover,

Table 2. Results of Slight Digestion Technique in Secondary Cultures

	Island-like growth of Epitheloid cell among Fibroblastic cells	
Success of Slight Digestion for	the elimination of F-type cells 16/20	the harvesting of F-type cells 20/20
Success of Sub-culturing of	E-type cells (remained) 4/16	F-type cells (harvested) 18/20
Remarks and Evaluations	Elimination is sometimes insufficient, Scratching must be added.	Some of E-type cells came off together, better harvest expected in the Subcultures.

Numerator ..... Successful results
Denominator ..... Expeirmental cases
E-type cells ..... Epitheloid cells
F-type cells ..... Fibroblastic cells

when intermingling fibroblastic cells appeared in the flask containing epitheloid cells, better growth of the epitheloid cells than in the pure situation was noted. This suggests that epitheloid cells are metabolically dependend on fibroblastic cells. Therefore, media which consist of half fresh MEM with 20 % fetal calf serum and half old 199 with 20 % fetal calf serum decanted from fibroblastic cells is recommended for use in the epitheloid cell cultures. Sometimes good results could be obtained by this method.

Scratching technique (for picking up) in both primay and secondary cultures

Results of this technique applied to the island-like growth of epitheloid cells among fibroblastic cells are shown in Table 3. Subculturing of both epitheloid and fibroblastic cells was sometimes successful ; however, the latter was better than the former in general. Cells which were picked up usually clumped when they were transferred into new flasks with fresh media. However, clumps of the fibroblastic cells usually showed good growth and formed a thick sheet. The epitheloid cells did not. From this experiment it was observed that fibroblastic cells seem to hold together well, and that they survive mechanical treatment better than epitheloid cells.

Table 3. Results of Scratching Technique for Picking up in Primary and Secondary Cultures

	Island-like growth of Epitheloid cells among Fibroblastic cells	
	E-type cells	F-type cells
Success of Picking up Technique	20/20	20/20
Success of Subculturing	5/20	15/20

Numerator ..... Successful results
Denominator ..... Experimental cases
E-type cells ..... Epitheloid cells
F-type cells ..... Fibroblastic cells

Scratching technique (for erasing) in both primary and secondary cultures

This technique was very successful. Results are showed in Table 4. In the case of fibroblastic cells, subculturing was usually successful ; but in the case of epitheloid cells subculturing was not as good as expected.

In this process, difficulty of detachment of epitheloid cells with ATV was also seen; the problem of low population was solved by using many flasks with abundant cells. Therefore, subculturing of the epitheloid cells was somewhat better ; that of the fibroblastic cells was always successful. However, this technique is inferior to the punching technique from the viewpoint of cell-purity.

Unnecessary cells or colonies in the hidden area or dead angle of the flasks (which could not be reached by the curved rubber policeman) often remained in the subculture.

Table 4. Results of Scratching Technique for Erasing in Primary and Secondary Cultures

	Solitary and Colonial Growth		Clustered and Colonial Growth		Island-like Growth of Epitheloid Cells among Fibroblastic Cells*	
Success of Scratching	E	F	E	F	E	F
	20/20	20/20	13/20	20/20	18/20	16/20
Success of Subculturing	F	E	F	E	F	E
	20/20	5/20	13/13	4/20	18/18	6/16

* This was done after slight digestion with ATV solution

Numerator ..... Successful results
Denominator ..... Experimental cases
E ..... Epitheloid cells
F ..... Fibroblastic cells

A combination of the picking-up and erasing techniques is sometimes recommended. The scratching is excuted on the necessary cells or colonies ; then they are harvested and transferred into a new flask. Then erasing of doubtful cells and colonies can be made in the original flask. Thus pure cultures of both epitheloid and fibroblastic cells can be obtained by the application of picking-up and erasing techniques in one flask.

Evaluation of these techniques

Application of each technique in the case of solitary colonial growth of one type cells is very effective, however, in the case of completely mixed growth of epitheloid cells and fibroblastic cells in a thick sheet formation, which is usually seen in secondary and maintenance cultures, each of them is not always suitable for the separation. Flasks with this kind of growth should be transferred into a

large number of flasks which would be expected to show colonial or islandlike growth of epitheloid cells among fibroblastic cells. Then elimination by scratching, picking-up with the use of a rubber policeman, or slight digestion with ATV are all available.

The best method recommended from the viewpoint of effeciency is the elimination of unnecessary cells or colonies by scratching with a rubber policeman, especially after slight digestion with ATV.

In general, none of the separation techniques was difficult ; however, subculturing of the separated cells, particularly of epitheloid cells, involved many problems. Factors such as suitable media, population size, and incubation term should be examined further.

Concerning culture media, MEM with 20% FCS has proven most successful in these experiments. Population size is also important as gradual multiplication is the most desirable. For example, the cells in one Leighton tube can be transferred into two tubes. Transferral into 4 tubes might be dangerous. The incubation term should be less than 2 weeks when subculturing is successful. After an incubation of more than 3 weeks, epitheloid cells become somewhat resistant to ATV treatment, and fibroblastic cells begin to degenerate.

Cell type and transformation of cultured mink cells

After separation, the established cell strains of epitheloid cells and fibroblastic cells are fairly pure (Photo. 4, 5). However, the reappearance of fibroblastic cells during subculturing of epitheloid cells is frequently observed. This phenomenon sometimes results from incomplete separation and elimination of fibroblastic cells. Therefore, elimination of unnecessary cells must be always be accomplished in the maintenance culture, even after the establishment of a cell strain.

An amoeba-like cell, a kind of intermediate form, is also observed in the primary and maintenance cultures. The appearance of this type at the peripheral area of fibroblastic growth was noticed in earlier days[10], and was confirmed even at the peripheral area of epithelial growth in the present experiment. This type of cells was sometimes seen mingled among the separated cells (Photo. 6). They were very difficult to eliminate during subculturing ; however, they can be ignored due to their relatively small number.

The appearance of an intermediate type of cell other than the amoeba type cell was also very frequent ; however, coexistence of these cells in the fibroblastic cells can be permitted for two reasons : their number is very small, and they resemble the fibroblastic cells at the present time.

The cell types, particularly shapes and identification, are a complicated problem in cultured cells, as Willmer[11] noted in his review. The origin of epitheloid cells is believed to be the macrophages in tissue culture of peritoneal exudate ; in general, however, transformation of cells from one type to another also presents

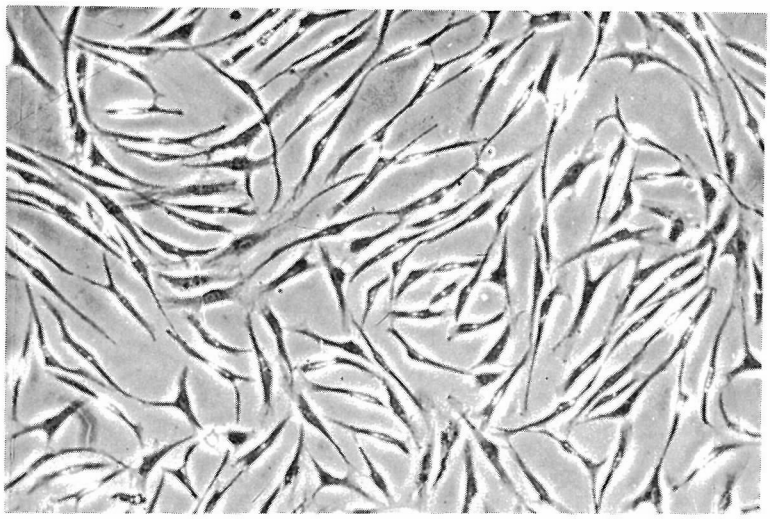


Photo. 4. Separated cell strain of Fibroblastic cell, Magnification 135×.

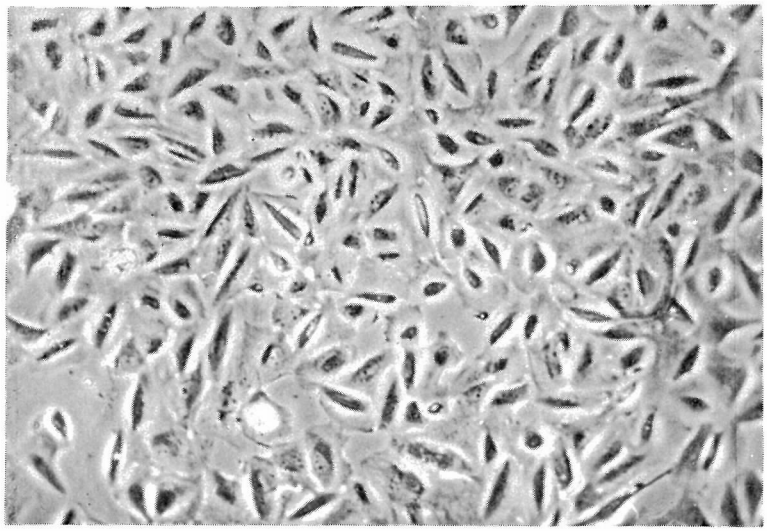


Photo. 5. Separated cell strain of Epitheloid cell in clustered growth, Magnification 135×.

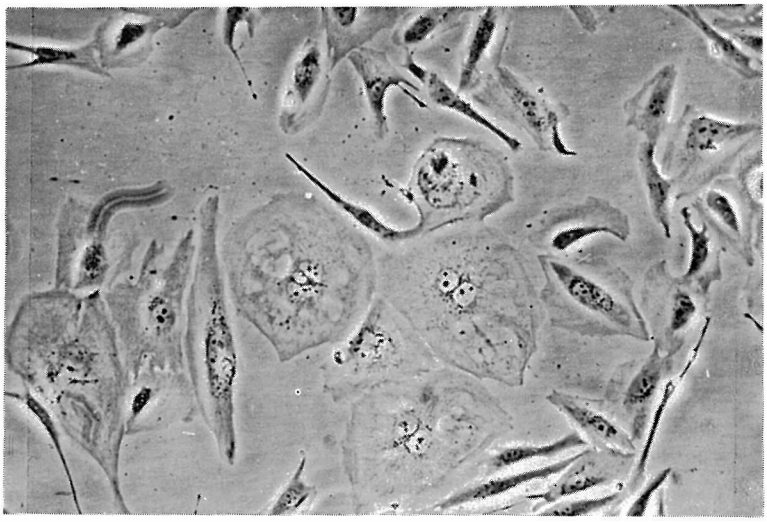


Photo. 6. Some Amoeba-type cells among Epitheloid cells, Magnification 135×.

a problem. Jacoby[12] noted that transformation from macrophages to fibroblastic cells and from fibroblastic cells to macrophages is seen in macrophage cultivation. These descriptions show confusion in identification of our cultured cells ; however, the authors want to limit the range of study to the separation methods. Identification will be discussed in an another paper.

ACKNOWLEDGEMENT

The authors wish to thank Mrs. Shirley D. Johnston, for her assistance on this study.

REFERENCES

1) Parker, R.C.: Personal Communication (1969)
2) Parker, R.C.: Cultivation of Epithelial Cells in Plasma. *Methods of Tissue Culture*, 3rd Ed., Paul B. Hoeber. Inc. N.Y. 164–165, 1961.
3) Baker, L.E.: Artificial media for the cultivation of fibroblasts, epithelial cells and monocytes. *Science*, **83**: 605–606, 1936.
4) Weiss, L.P. and Fawcett, D.W.: Cytochemical observations on chicken monocytes, macrophages and giant cells in tissue culture. *J. Histochem. Cytochem.*, **1**: 47–65, 1953.
5) Abercrombie, M. and Ambrose, E.J.: The surface properties of cancer cells, A Review. *Cancer Res.*, **22**: 525–548, 1962.
6) Heaton, J.B.: The nutritive requirements of growing cells. *J. Path. Bact.*, **29**: 293–306, 1926.
7) Medaware, P.B.: A factor inhibiting the growth of mesenchyme. *Quart. J. exp. Physiol.*, **27**: 147, 1937.

8) Medaware, P.B., Robinson, G.M. and Robinson, R.: A synthetic differential growth inhibitor. *Nature*, London, **151**,: 195, 1943.
9) Padgett, G.A., Leader, R. W., Gorham, J.R. and Omary, C.C.: The familial occurance of the Chediak-Higashi syndrome in mink and cattle. *Genetics*, **49**: 505–512, 1964.
10) Kapel, O.: Einige Untersuchungen über des Verhalten des Epithels in vitro. *Arch. exp. Zellforsch.*, **8**,: 35–129, 1929.
11) Willmer, E.N.: Morphological Problems of Cell Type, Shape and Identification. *Cells and Tissues in Culture* edited by E.N. Willmer, Academic Press (London & New York), Vol. I, 143–176, 1965.
12) Jacoby, F.: Macrophages. *A Review in Cells and Tissues in Culture*, edited by Willmer, E.N., Academic Press (London & NewYork), Vol. I, 1–78, 1965.